Drosophila Olfactory Neuroecology
-Function and Evolution

Dissertation

To Fulfill the
Requirements for the Degree of
Doctor of Philosophy (Ph.D.)

Submitted to the Council of the Faculty
of Biology and Pharmacy
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INTRODUCTION

Animals use a wide range of sensory systems to locate and evaluate food, shelter, mates, and breeding substrates as well as to avoid predators and other dangers, and thus providing the nervous system with information to map features of the external world into internal representations that allow animals to navigate their environments and locate resources.

Deciphering neural correlates of natural behaviors associated with an animal's ecological niche is termed neuroecology (Purves and Lichtman, 1985) and has been approached in various ways. Since the molecular and cellular logic of odor coding has been largely deciphered in most established model systems, such as the fly and the mouse, olfactory neuroecology has become a hot issue in neuroscience (reviewed in Brennan and Zufall, 2006; Luo and Flanagan, 2007; Laissue and Vosshall, 2009; Hansson and Stensmyr, 2011).

Insects are the most diverse class of animals on earth, with approximately one million described species occurring across a wide variety of lifestyles and habitats and displaying a plethora of diverse behaviors (Grimaldi and Engle, 2005). The relatively simple organization of neuronal circuits along with the short life time and high reproductive rate make insects ideal for neuroecological studies.

Drosophila as a model for studying olfactory neuroecology

From the over 1500 species in the genus *Drosophila* that have so far been described (Grimaldi, 1990; Powell, 1997; Ashburner, 2005; Markow and O'Grady, 2006), only one species, the common vinegar fly, *Drosophila melanogaster*, has for over a century been used as a model organism in scientific research. *Drosophila* came to be a central organism in genetics in the early 1900's, when Thomas Hunt Morgen at Columbia University was looking for a suitable species in which to perform studies of heredity. The intense studies of him and his talented students, Calvin B. Bridges, Herman J. Muller and Alfred H. Sturtevant, established most of the major principles of classic genetics such as the chromosome theory of heredity, the nature of genetic linkage and genetic maps, the genetic behavior of chromosome aberrations, the induction of gene and chromosome mutations by radiation, the discovery of mitotic recombination, and so forth (Sturtevant, 1965). Discoveries for which Morgan and Muller received the Nobel prize in medicine in 1933 and 1946 respectively. These groundbreaking studies were followed by many major technical advances in the field, such as the invention of the p-element based transformation technology (Kidwell et al., 1977; Rubin

and Spradling, 1982; Spardling and Rubin, 1982), the powerful methods for clonal analysis (Cooley *et al*., 1988), and the discovery of potent chemical mutagens (Lewis and Bacher, 1968).

Drosophila melanogaster thus offers many unique advantages for studying olfactory neuroecology as a host of sophisticated manipulations can be carried out (Venken and Bellen, 2005, 2007). Mutations can be engineered in the locus of interest through selective removal or replacement of sequences (Rong et al., 2002), the expression level of any gene can be reduced using RNA interference (Dietzl et al., 2007; Ni et al., 2009), any gene can be ectopically express in almost any tissue or cell using the yeast Gal4-UAS system (Brand and Perrimon, 1993), and high precision manipulations in the genome can even be performed using the Flp-FRT recombination system (Golic and Lindquist, 1989). In addition, thousands of UAS/Gal4 lines are now available (Pfeiffer et al., 2008), which allow for modification of gene expression (Brand and Perrimon, 1993), or to functionally or physically ablate most neuronal populations in the brain (Sweeney et al., 1995; Kitamoto, 2000; Ren et al., 2001; Luan et al., 2006). Moreover, a recent development is the optogenetic and thermogenetic tools. These allow stimulation of specific neural populations and their connected innate behaviors under the control of a light or temperature sources, respectively (Miesenbock, 2009). Finally, 21 *Drosophila* species genomes have up to now been sequenced (http://flybase.org/blast/). These species evolved and diverged during the last 63 million years (MY) (Tamura et al., 2004), thus providing a strong background for comparative studies within a tightly defined molecular and phylogenetic framework.

***Drosophila*-microbe-plant interactions**

Drosophila species are primarily consumers of microorganisms, including yeast and bacteria, which are in turn often associated with the initial stages of decay of plant materials, such as fruit, flowers, tree saps, barks, leaves, or fleshy fungi (Carson, 1971). Some species are known to feed and breed on fresh or living plant materials, such as flowers (Sturtevant, 1921; Brncic, 1966; Pipkin et al., 1966) and leaves (Okada, 1968), and fruit (Lachaise, 1977). Some other species have evolved other, very special feeding habits; such as breeding on/in land crabs (Carson, 1974) and spider's eggs (Heed, 1968).

Yeasts are the major source of nutrition for adults and larvae of most *Drosophila* (Diptera: Drosophilidae) (Begon, 1982), and larval growth and survivorship is affected by the yeast

species available to them (Starmer and Aberdeen, 1990). Yeast species may differ in composition and quality, depending on both the yeast itself and the substrate on which the yeast grows (Ganter, 2006). A higher nutritional value of fruit inoculated with yeast over fruit alone is expected to favour sensory and physiological adaptations that facilitate detection and location of fermenting fruit for feeding and egg-laying. Yeasts are known for producing a wide range of secondary metabolites including volatile organic compounds, and there is growing evidence that these compounds have distinct ecological functions (reviewed in Davis *et al*., 2013). For example, some of the microbial volatile organic compounds attract or repel insects, inhibit the growth of microorganisms competing with associated insects, stimulate oviposition, mimic plant hormones, or even induce plant resistance (Davis et al., 2011; Ryu et al., 2003, 2004).

Thus, *Drosophila* species that are immersed in thousands of good and bad odor signals in their natural habitat need sophisticated olfactory systems to detect and interpret these signals. Moreover, the olfactory systems must be able to quickly extract relevant information from an enormously complex external environment.

Structures involved in *Drosophila* olfaction

As in most other insects, *Drosophila* senses volatile chemicals with two pairs of olfactory organs located on the head. These olfactory appendages are the third antennal segment, in the fly termed the funiculus, and the maxillary palps (Figure 1A). These are both densely covered with porous hair-like structures, so-called olfactory sensilla, which house the dendrites of one to four olfactory sensory neurons (OSNs) in four distinct morphological sensillar types: club-shaped basiconic, long and pointed trichoid, intermediate and short, peg-like coeloconic sensilla (Shanbhag et al., 1999) (Figure 1C and D). The cell bodies of the OSNs are surrounded by three auxiliary cells; the thecogen, tormogen, and trichogen cells, which are involved in the formation of the sensillum during ontogeny, as well as maintaining the ionic composition of the sensillum lymph (Figure 1B) (Hansson, 1995), e.g. the synthesis of the so-called odorant binding proteins (OBPs, Vogt and Riddiford, 1981). These OBPs are probably involved in transporting the mostly hydrophobic odor ligands through the aqueous sensillum

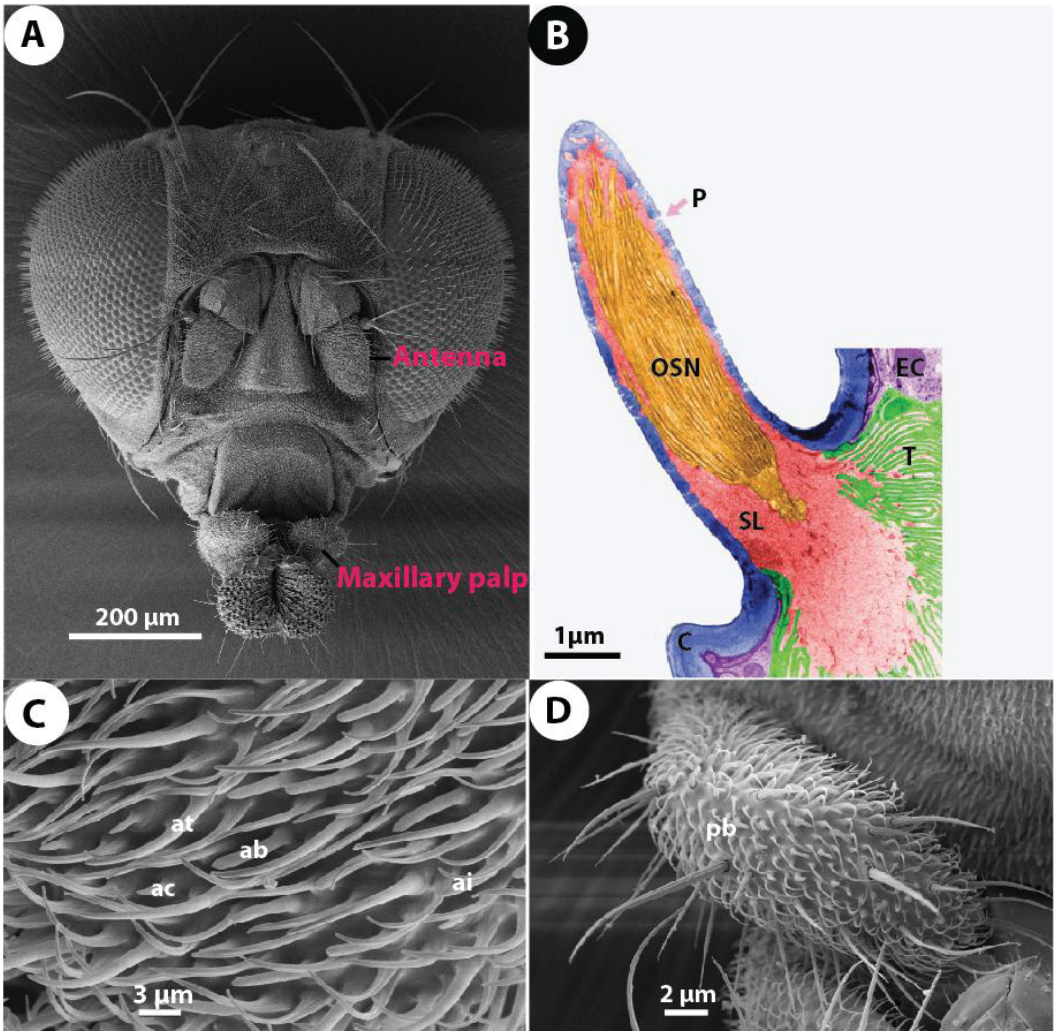


Figure 1. *Drosophila* peripheral olfactory organs. (A) The head capsule with the antennae and the maxillary palps. (B) Olfactory sensillum (modified after Hansson and Stensmyr, 2012), C = Cuticle, EC = Epidermis cell, OSN = olfactory sensory neuron, P = Pore, SL = Sensillum lymph, and T = Tormogen/trichogen cell (C) Four morphological types of olfactory sensilla on the antennal surface: ab = antennal basiconic, at = antennal trichoid, ai = antennal indermedia and ac = antennal coeloconic. (D) One morphological type of the maxillary palp olfactory sensilla, pb = palp basiconic.

lymph to the receptor sites. These proteins are situated in the dendritic membrane of the OSNs (Laughlin et al., 2008). In addition, other protein types of different functions have also been found in the sensillum lymph (e.g. chemosensory proteins (CSPs) (Vogt, 2003; Pelosi et al., 2006)) and odor degrading enzymes (ODEs) (Vogt, 2003).

The third antennal segment is covered with all four types of sensilla, basiconic, trichoid, intermediate and coeloconic (Figure 1C), which are in turn further subdivided in a stereotyped distribution and bilaterally symmetric pattern with large basiconic sensilla clustered at the medial-proximal side of the antenna and trichoid sensilla clustered at the lateral-distal edge (Shanbhag et al., 1999). Intermediate sensilla, which combine features of basiconic and trichoid sensilla, are found intermingled among the latter. Small basiconic and coeloconic sensilla are interspersed in the middle region of the antenna. In total, there are between 1100–1250 OSNs in each antenna, with roughly 20% fewer large basiconic and 30% more trichoid sensilla in males than females (Stocker, 2001). These four sensillar types serve distinct chemosensory functions as they contain OSNs responding to different types of chemical stimuli. Basiconic OSNs respond to general odorants, trichoid OSNs respond exclusively to pheromones (Clyne et al., 1997; Hallem and Carlson, 2006; van der Goes van Naters and Carlson, 2007) and coeloconic OSNs respond to a variety of amines and carboxylic acids (Benton et al., 2009; Yao et al., 2005), while the ligand specificity of the OSNs housed in intermediate sensilla was unknown until recently (see chapter II of this thesis, Dweck et al., 2013).

The club-shaped maxillary palp is inserted at the base of the proboscis. The palps are hidden between the retracted proboscis and the head capsule at rest and during flight, and are exposed only when the proboscis is extended (Shanbhag et al., 1999). The palps are simpler olfactory organs, containing sixty basiconic sensilla (Figure 1D) each housing two OSNs. Most of the palp sensilla are located on the distal half of the dorsal and on the lateral edges. A few sensilla are also found on the ventral surface of the palp (Singh and Nayak, 1985). Based on the branching pattern of the outer dendritic segments of OSNs, the palp sensilla can further be divided into three subtypes, PB‐I, PB‐II and PB‐III (Shanbhag et al., 1999). The PB‐I OSNs contain highly branched terminal dendrites, while the PB‐II OSNs are characterized by ribbon‐shaped dendrites. Lastly, the PB‐III OSNs are rarer on the palp and have an unusually thick, hollow dendritic segment.

Molecular basis of *Drosophila* olfaction

Insects have evolved large numbers of receptors that belong to multiple families in order to facilitate detection and discrimination of the vast number of odorants that are encountered in their environments (Figure 2). Insect genomes contain 60–340 members of the phylogenetically distinct insect Odorant receptor (Or) family (Touhara and Vosshall, 2009). In addition, a few members of the Gustatory receptor (Gr) family are expressed in olfactory organs, where some have been found to mediate response to CO_2 (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004). Recently, another family of ~ 66 genes called Ionotropic receptors (Irs) has been identified, of which several are expressed in OSNs of coeloconic sensilla (Benton et al., 2009, Croset et al., 2010).

Odorant receptors were first discovered in the rat by Linda Buck and Richard Axel in 1991. Later, in 1999, three independent groups identified the first insect Ors in *D. melanogaster* by using different approaches (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). The Or family of both vertebrates and insects encode receptor proteins with seven transmembrane spanning domains, but in insects, the membrane topology is inverted with N-termini located in the cytoplasm and C-termini located extra-cellularly (Benton et al., 2006; Wistrand et al., 2006). Another contrary to the vertebrate Or family is that the insect Or family forms heteromeric complexes composed of a single ligand-binding Or (Störtkuhl and Kettler, 2001; Dobritsa et al., 2003; Goldman et al., 2005) and the Or co-receptor Orco (in *Drosophila* formerly termed Or83b) (Figure 2A) (Vosshall et al., 2000; Larsson et al., 2004). Orco acts as a chaperone protein (Larsson et al., 2004; Benton et al., 2006)), and takes part in signal transduction (Sato et al., 2008; Wicher et al., 2008).

The *D. melanogaster* Or family contains 60 genes, where two are alternatively spliced, resulting in 62 Or genes in total (Clyne et al. 1999; Vosshall et al. 1999; Roberstson et al., 2003; Couto et al. 2005). Of this total, 39 Or genes are expressed only in the adults, and are found across 3 antennal trichoid, 10 antennal basiconic, and 3 palp basiconic sensillum types (Vosshall et al., 2000). In addition, 25 Or genes are expressed only in the larval olfactory organ, the dorsal organ (Fishilevich et al., 2005), and 12 Or genes are expressed in the adult as well as the larva.

Another more recent addition to the olfactory arsenal of *Drosophila* are the Irs. A genomic analysis identified 66 Ir genes of three transmembrane domains and a pore loop in *D.*

melanogaster (Benton et al., 2009; Croset et al., 2010), either uniquely, or co-expressed with one or two other Irs, specifically Ir8a and/or Ir25a. Sixteen of these genes are expressed in the antenna and ten are expressed in 4 selective subsets of coeloconic sensillum OSNs (ac1-ac4) (Yao et al., 2005; Benton et al., 2009; Croset et al., 2010). One neuronal class found in the ac3 sensillum co-expresses Or35a and Ir76b, but the characterized odor responses in these neurons depend solely on the Or gene (Benton et al., 2009; Yao et al., 2005). The four remaining antennal Irs (Ir21, Ir40a, Ir64a, and Ir93a) are found in arista and sacculus neurons (Benton et al 2009; Ai et al., 2010).

Most of the *Drosophila* Ors and Irs have now been functionally characterized using the single sensillum recording (SSR) technique and the empty neuron system. The SSR technique is used to determine the odorant specificity and sensitivity of a single OSN in any sensillum and is performed by using two sharpened tungsten electrodes: the ground electrode, which is inserted into the eye and the recording electrode that is gently inserted into the base of a single sensillum. The generated action potential is amplified, passed on to a digital converter and the spikes recorded and analyzed via computer software. Application of odorants can either increase the OSN action potential frequency (excitation) or reduce the spontaneous action potential rate (inhibition). The SSR technique has allowed researchers to generate an almost complete coding map of the peripheral olfactory organs of the fly (Table 1) (de Bruyne et al., 1999, 2001; Stensmyr et al., 2003; Hallem and Carlson, 2006, Silbering et al., 2011, Stensmyr et al., 2012; Dweck et al., 2013).

The empty neuron system is used to determine the response profile of a specific Or. The system involves the Δhalo mutant, which lacks Or22a/b but retains expression of Orco (Dobrista et al., 2003; Hallem et al., 2004; Hallem and Carlson, 2006) in the ab3A OSN. Different Or genes can be expressed by transgenic techniques in this “empty neuron” and the Or response profile can be measured directly without interference from the resident Or. Thus, by comparing the odor response spectra conferred by individual Ors with the odor response spectra of wild-type OSNs, many Ors have been mapped to the OSNs from which they are derived (Table 1) (Dobrista et al., 2003; Hallem et al., 2004; Couto et al., 2005; Goldmann et al., 2005; Hallem and Carlson, 2006).

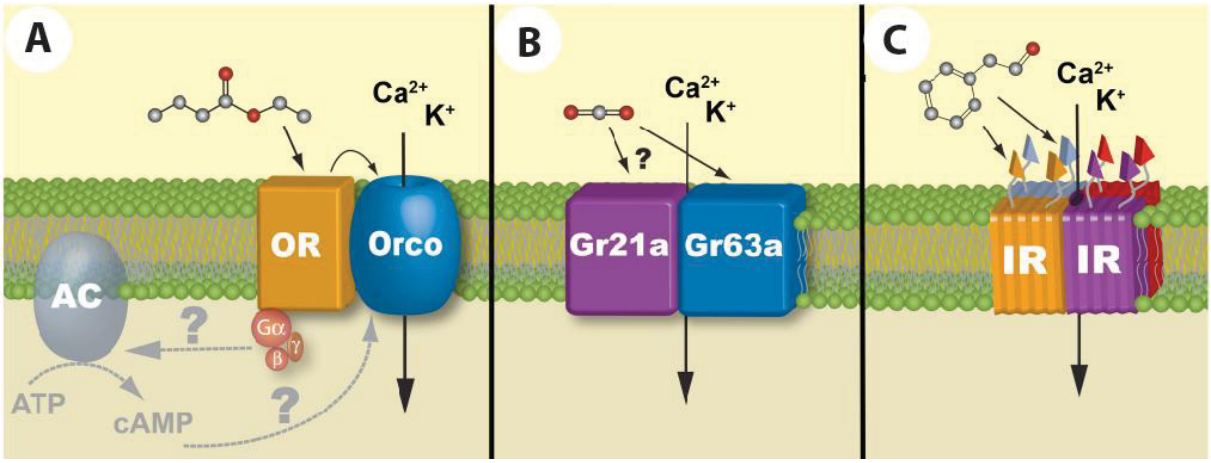


Figure 2. Odorants are detected through diverse signaling mechanisms in *Drosophila*. (A) A 'tuning' OR forms a heterodimer with the ion channel Orco. Food odorants (shown as the ball and stick structure) interact with the 'tuning' OR, which in turn activates the Orco ion channel. Orco conducts potassium and calcium ions into the olfactory neurons, resulting in depolarization and initiation of action potentials. Odorant binding to the OR may also trigger activation of Orco via a second messenger, wherein a G protein (in red) stimulates adenylyl cyclase (AC) to produce cAMP, which in turn activates Orco. (B) CO_2 detection is mediated by a heterodimer of gustatory receptors Gr21a and Gr63a that are expressed in ac1c basiconic OSNs of the antenna. (C) Variant ionotropic glutamate receptors (Irs), mediate odorant detection in coeloconic sensilla. The extracellular ligand-binding domains of Irs (tethered triangles) likely recognize odorants and activate the channels, which are likely to be heteromultimers (Ronderos and Smith, 2009).

Sensillum type	Sensillum subtype	Neuron	Receptor(s)	Glomerulus	Best ligand
antennal basiconic	ab1	ab1A ab1B ab1C ab1D	Or42b Or92a Gr21a/Gr63a Or10a/Gr10a	DM1 VA2 V DL1	Ethyl acetate Ethyl lactate* Carbon dioxide Methyl salicylate
	ab2	ab2A ab2B	Or59b Or85a	DM4 DM5	Methyl acetate Ethyl 3-hydroxybutyrate
	ab3	ab3A ab3B	Or22a/Or22b Or85b	DM2 VM5d	Ethyl hexanoate 2-heptanone
	ab4	ab4A ab4B	Or7a Or33a/Or56a	DL5 DA2	E2-hexenal **Geosmin**
	ab5	ab5A ab5B	Or82a Or47a/Or33b	VA6 DM3	Geranyl acetone pentyl acetate
	ab6	ab6A ab6B	Or13a Or49b	DC2 VA5	1-octen-3-ol Guaiacol*
	ab7	ab7A ab7B	Or98a Or67c	VM5v VC4	Ethyl benzoate Ethyl lactate
	ab8	ab8A ab8B	Or43b Or9a	VM2 VM3	Ethyl trans-2-butenoate 3-hydroxy-2-butanone
	ab9	ab9A ab9B	Or69a/Or69b Or67b	D VA3	**Citral** 2-phenylethanol
	ab10	ab10A ab10B	Or67a Or49a/Or85f	DM6 DL4	Benzyl butyrate* unknown
antennal intermediate	ai1	ai2A ai2B	Or83c Or23a	DC3 DA3	Farnesol* unknown
	ai2	ai3A ai3B ai3C	Or19a/Or19b Or2a Or43a	DC1 DA4m DA4l	**Valencene** unknown Methyl indole*
antennal trichoid	at1	t1A	Or67d	DA1	11-cis vaccenyl acetate
	at4	t4A t4B t4C	Or47b Or65a/Or65b/Or65c Or88a	VA1v DL3 VA1d	Unknown Unknown Unknown
antennal coeloconic	ac1	ac1A ac1B ac1C	Ir31a Ir75d Ir92a/Ir76b	VL2p VL1 VM1	2-oxopentanoic acid Pyrrolidine Ammonia
	ac2	ac2A ac2B ac2C	Ir75a Ir75d Ir41a/Ir76b	DP1l VL1 VC5?	Acetic acid Pyrrolidine 1,4-diaminobutane
	ac3	ac3A ac3B	Ir75c/Ir75b/Ir75a OR35a/Ir76b	DP1l VC3	Butyric acid 1-hexanol
	ac4	ac4A ac4B ac4C	Ir84a Ir75d Ir76a/Ir76b	VL2a VL1 DC4	Phenylacetic acid Pyrrolidine Phenylethylamine
palp basiconic	pb1	pb1A pb1B	Or42a Or71a	VM7 VC2	Gamma-hexalactone* 4-ethylguaiacol*
	pb2	pb2A pb2B	Or33c/Or85e Or46a	VC1 VA7l	beta-Ionone* Phenol*
	pb3	pb3A pb3B	Or59c Or85d	1 VA4	Benzyl cyanide* 2-phenylethyl acetate*

Table 1. *Drosophila* sensillum types and subtypes, their OSN names, their Or genes expressed, their glomeruli innervated, and their best ligands. Ligands in bold were characterized in the present thesis. Asterisks indicate unpublished data.

How is the olfactory sensory information represented in the *Dropsophila* brain?

The OSNs from the antenna and the maxillary palp send axons into the brain. There, they target the insect primary olfactory center, the antennal lobe (AL) (Figure 3), which is the equivalent of the olfactory bulb in vertebrates (Strausfeld and Hildebrand, 1999; Ache and Young, 2005). As in vertebrates, the AL is composed of spheroid structures, called glomeruli. OSNs expressing the same Or project their axons to one of ~ 50 stereotypic glomeruli in the AL, where axonal branches synapse with dendrites of the corresponding class of projection neurons (PNs) (Vosshall et al., 2000; Goa et al., 2000; Bhalerao et al., 2003; Wilson and Mainen, 2006; Maresh et al., 2008). The PNs mostly innervate one glomerulus but some innervate multiple. The glomeruli of the AL are also innervated by local interneurons (LNs) that non-uniformly innervate most of the glomeruli of the AL (Stocker et al., 1990; Wilson and Laurent, 2005; Seki et al., 2010) and are thought to sharpen the input into the AL via inhibitory interactions (Wilson, 2013). The axons from uniglomerular excitatory PNs (ePNs) relay olfactory information to the mushroom body (MB), a center for olfactory learning and memory (Davis, 2005; Heisenberg, 2003), and to the lateral horn (LH), a less-understood higher-order center presumed to direct olfaction-mediated innate behavior (Heimbeck et al., 2001), while the axons of the oligoglomerular inhibitory PNs (iPNs) target the LH exclusively (Liang et al., 2013).

The glomeruli targeted by axons of neurons expressing specific receptors were furthermore mapped by driving the expression of membrane-bound GFP with Or promoters (Vosshall et al., 2000; Couto et al., 2005; Silbering et al., 2011). By analyzing the complete Or and Ir families in this manner, a map of the *Drosophila* AL was constructed (Table 1). This map established the chemical specificity of individual glomeruli in the AL and of the projection neurons that relay glomerular activity to the higher olfactory processing centers. The combination of detailed functional and anatomical characterization of nearly all the olfactory neuron classes makes the *Drosophila* olfactory system one of the best where to study olfactory neuroecology.

During my thesis I wanted to study the ecological functions of *Drosophila* Ors because when I started in 2010, there had been only one investigation on the olfactory pathway; the one that underlies female courtship receptivity in the vinegar fly, *Drosophila melanogaster* (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007). Therefore, in chapter I, we identified a functionally segregated olfactory circuit in flies that is activated exclusively by the microbial odor, geosmin, to alert flies to the presence of harmful microbes (Stensmyr et al., 2012). In chapter II, we demonstrated that a single dedicated olfactory pathway

determines oviposition fruit substrate choice (Dweck et al., 2013). Finally in chapters IV and V, we begin to unravel the olfactory mechanisms underlying intraspecific divergence and the evolution of host-plant specialization in *D. erecta and D. mojavensis* (Linz et al., 2013; Date et al., 2013).

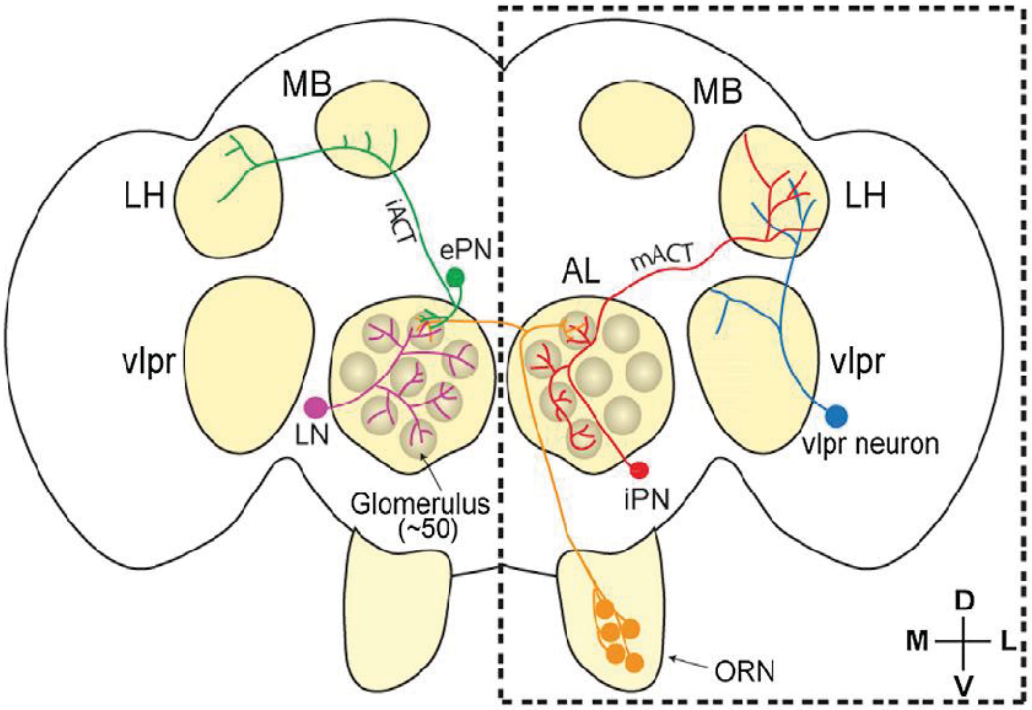


Figure 3. Schematic diagram of the fly olfactory system. OSNs (orange) send their axons to the antennal lobe (AL) in both hemispheres and synapse with dendrites of projection neurons (PNs) and local interneurons (LNs, purple). Excitatory PNs (ePNs, green) project their axons through the inner antennocerebral tract (iACT) to the mushroom body (MB) and lateral horn (LH). Inhibitory PNs (iPNs, red) send axons through the middle antennocerebral tract (mACT) to innervate the LH only. Also shown is a class of putative third-order neurons (blue) that connects the LH with the ventrolateral protocerebrum (vlpr). D, dorsal; V, ventral; M, medial; L, lateral. (modified after Liang et al., 2013).

OVERVIEW OF MANUSCRIPTS

This thesis is based on the following manuscripts:

Manuscript I

A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in *Drosophila*

Marcus C. Stensmyr*, Hany K.M. Dweck* Abu Farhan*, Irene Ibba*, Antonia Strutz, Latha Mukunda, Jeanine Linz, Veit Grabe, Kathrin Steck, Sofia Lavista-Llanos, Dieter Wicher, Silke Sachse, Markus Knaden, Paul G. Becher, Yoichi Seki, and Bill S. Hansson

* These authors contributed equally to the work.

Cell, 2012, 151(6):1345-1357

Here, we describe a functionally segregated olfactory circuit in the vinegar fly, *D. melanogaster* that is exclusively activated by geosmin to alert flies to the presence of harmful microbes. Geosmin active only a single class of sensory neurons, ab4B, expressing the olfactory receptor Or65a. Selectively silencing Or56a-neurons abolishes the avoidance behavior to geosmin, suppresses the aversive influence of this compound on feeding, and flies lay eggs upon medium containing *S. coelicolor* as well as uncontaminated medium. Expressing the temperature sensitive dTRPA1 in Or56a neurons is also sufficient to make flies to avoid blue light. Geosmin also overrides and modulates innate attraction to vinegar, which confers obligate attraction in flies.). Moreover, the geosmin detection system was highly conserved across species in the genus *Drosophila*, suggesting that the circuit evolved to enable avoidance of toxic feeding and breeding sites in the environment.

Built on an idea conceived by all authors.
Designed experiments: MCS, BSH, HKMD
Performed experiments: SSR (100%), GC-SSR (100%), Behavior and Genetics (50%)
Data analyses: SSR (100%), GC-SSR (100%), Behavior and Genetics (50%).
Wrote manuscript: MCS, BSH, HKMD

Manuscript II

Olfactory Preference for Egg Laying on Citrus Substrates in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Sophie Kromann, Deni Bown, Ylva Hillbur, Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

Current Biology, 2013, 23:1-9

We demonstrate that flies prefer Citrus fruits as oviposition substrate and this preference is mediated via a single class of olfactory sensory neurons, dedicated to the detection of terpenes typical of flavedo (i.e., the colored rind found in Citrus). The Citrus partiality likely reflects an ancestral preference toward specific fruits found in the native African habitat. Finally, we demonstrate that the Citrus preference has likely been driven by needs to avoid parasitization from endoparasitoid wasps.

Built on an idea conceived by all authors.
Designed experiments: MCS, BSH, HKMD
Performed experiments: SSR (100%), GC-SSR (100%), Behavior and Genetics (50%)
Data analyses: SSR (100%), GC-SSR (100%), Behavior and Genetics (50%).
Wrote manuscript: MCS, BSH, HKMD

Manuscript III

Host Plant-Driven Sensory Specialization in *Drosophila erecta*

Jeanine Linz, Amelie Baschwitz, Antonia Strutz, Hany K. M. Dweck,

Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

Proceedings of the Royal Society B: Biological Science, 2013, 280: 20130626

We show that in comparison with three sympatric sibling species, the olfactory system of *D. erecta* is more sensitive towards a characteristic *Pandanus* volatile, 3-methyl-2-butenyl acetate and this increased sensitivity is due to a numerical increase of one olfactory sensillum, ab3. Furthermore, we show that this numerical increase of this sensillum type is also reflected in the primary olfactory center, the antennal lobe. Finally, we show that the characteristic *Pandanus* volatile, 3-methyl-2-butenyl acetate trigger oviposition in *D. erecta* but not in *D. melanogaster*.

Built on an idea conceived by all authors.

Performed experiments: SSR and neuronal backfill (100%)

Manuscript IV

Divergence in Olfactory Host Plant Preference in *D. mojavensis* in Response to Cactus Host Use

Priya Date*, Hany K. M. Dweck*, Marcus C. Stensmyr, Jodi Shann, Bill S. Hansson, and Stephanie M. Rollmann

* These authors contributed equally to the work.

PLoS One, 8(7): e70027

We show divergence in electrophysiological responses and olfactory behavior among populations with host plant shifts. Specifically, we show that the Mojave Desert population significant differs in electrophysiological responses of the olfactory organs and in behavioral responses to its barrel cactus volatiles. Together our results suggest that the peripheral nervous system has changed in response to different ecological environments and that these changes likely contribute to divergence among *D. mojavensis* populations.

Built on an idea conceived by all authors.
Designed experiments: PD, HKMD, MCS, JS, BSH, SMR
Performed experiments: EAD (100%)
Data analyses: EAD (100%)
Wrote manuscript: PD, HKMD, MCS, SMR

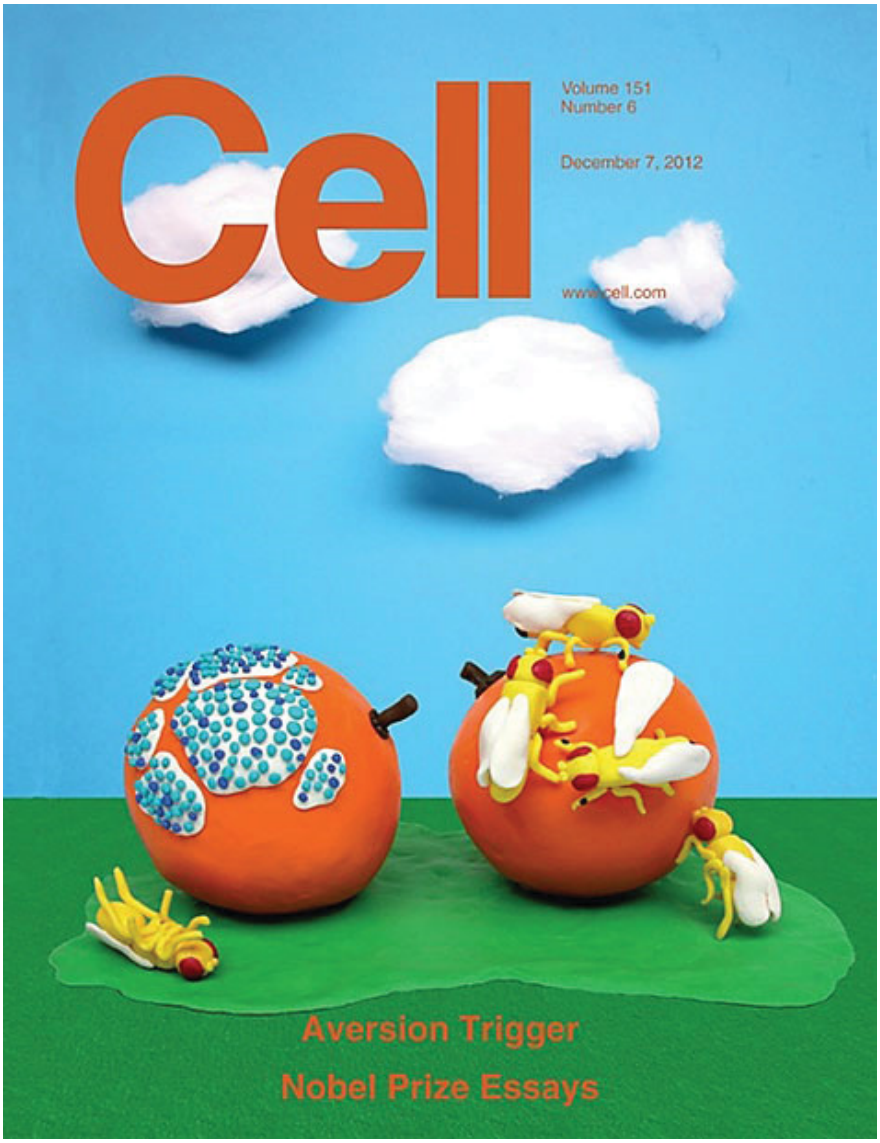
MANUSCRIPT I

A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in *Drosophila*

Marcus C. Stensmyr*, Hany K.M. Dweck* Abu Farhan*, Irene Ibba*, Antonia Strutz, Latha Mukunda, Jeanine Linz, Veit Grabe, Kathrin Steck, Sofia Lavista-Llanos, Dieter Wicher, Silke Sachse, Markus Knaden, Paul G. Becher, Yoichi Seki, and Bill S. Hansson

* These authors contributed equally to the work.

Cell, 2012, 151(6):1345-1357



Cell

A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in *Drosophila*

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SUMMARY

Flies, like all animals, need to find suitable and safe food. Because the principal food source for *Drosophila melanogaster* is yeast growing on fermenting fruit, flies need to distinguish fruit with safe yeast from yeast covered with toxic microbes. We identify a functionally segregated olfactory circuit in flies that is activated exclusively by geosmin. This microbial odorant constitutes an ecologically relevant stimulus that alerts flies to the presence of harmful microbes. Geosmin activates only a single class of sensory neurons expressing the olfactory receptor Or56a. These neurons target the DA2 glomerulus and connect to projection neurons that respond exclusively to geosmin. Activation of DA2 is sufficient and necessary for aversion, overrides input from other olfactory pathways, and inhibits positive chemotaxis, oviposition, and feeding. The geosmin detection system is a conserved feature in the genus *Drosophila* that provides flies with a sensitive, specific means of identifying unsuitable feeding and breeding sites.

INTRODUCTION

Animals respond with innate behaviors to certain stimuli in their environment. Innate behaviors, in contrast to learned behaviors, are hardwired; i.e., confronted with a specific stimulus, the animal will respond with a stereotyped behavior (Tinbergen, 1951). Many innate behaviors are triggered by odors. Prime examples are pheromones (Karlson and Lüscher, 1959), which have been particularly well studied in insects. In the vinegar fly *Drosophila melanogaster*, the male-produced pheromone *cis*-vaccenyl acetate (cVA) activates a single class of olfactory sensory neurons (OSN), which provides input to a single glomerulus (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007) and a sexually dimorphic and functionally segregated circuit within the olfactory system (Datta et al., 2008; Ruta et al., 2010). In insects, odors associated with food or oviposition substrates can also elicit innate behaviors. The smell of vinegar confers obligate attraction in flies (Stökl et al., 2010). Although the vinegar odor activates a number of OSN classes, only a single glomerulus is sufficient and necessary for positive chemotaxis (Semmelhack and Wang, 2009). Pathways underlying hardwired attraction have thus been well characterized. Olfactory circuits mediating odorant-induced innate avoidance are, however, poorly understood. From an evolutionary perspective, being able to detect and respond quickly to harmful features in the environment should be an essential task for the olfactory system. In the fly, CO_2 elicits innate avoidance, which, like the attraction pathways, is mediated via a single glomerular circuit devoted exclusively to this stimulus (Suh et al., 2004). No dedicated avoidance circuit for an odorant sensu stricto (i.e., a volatile organic compound) has, however, been found in the fly or in any other insect. So far, all identified aversive odorants have activated multiple glomeruli (Knaden et al., 2012), and their identification depends on decoding of complex combinatorial glomerular activation patterns.

A volatile compound of interest in this context is geosmin (trans-1,10-dimethyl-trans-9-decalol) (Figure 1A). This substance is produced by a select number of fungi (Mattheis and Roberts, 1992), bacteria (Gerber and Lechevalier, 1965), and cyanobacteria (Jüttner and Watson, 2007) and to the human nose has a distinct and immediately recognizable earthy odor. A recent study found that addition of a small amount of geosmin reduced the attraction of flies to vinegar volatiles (Becher et al., 2010). Given its capacity to modulate innate attraction, this microbial volatile must be a very potent repellent and, as such, is possibly a candidate stimulus for a dedicated pathway for innate avoidance.

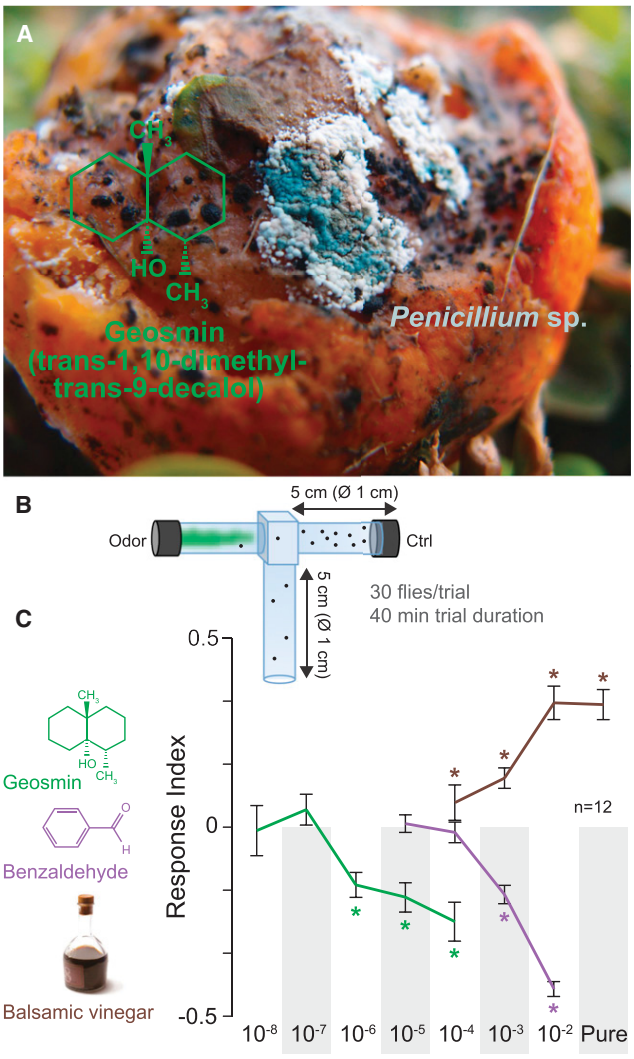

Figure 1. Geosmin—the Odor of Mold—Is Repellent to the Vinegar Fly

(A) Geosmin has a peculiar structure (left), which is distinct from odor ligands identified for *D. melanogaster*. Although a very common compound in nature, geosmin is produced only by a specific subset of microorganisms, including *Penicillium* sp. molds, shown here growing on an orange. Photo, MCS.

(B) Schematic drawing of the T-maze assay.

(C) Response indices of WT flies to geosmin, benzaldehyde, and balsamic vinegar in a T-maze assay. Deviation of the response index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

Here, we examine the functional significance of geosmin to the fly and show that geosmin activates only a single class of OSNs; these neurons express an odorant receptor that is exclusively tuned to this compound. Furthermore, we show that the geosmin-activated circuit constitutes a functionally segregated pathway, transferring the message arising from the periphery unaltered to central processing centers. We also demonstrate that this circuit alone is sufficient and necessary to trigger the avoidance behavior. Moreover, we show that, upon activation, the geosmin circuit overrides input from other circuits and inhibits positive chemotaxis. Additionally, we show that the peripheral part of the geosmin detection system is highly conserved across the genus *Drosophila*. Finally, we clearly demonstrate the ecological significance of this pathway, which is to detect toxic microbes.

RESULTS AND DISCUSSION

A Single Class of Olfactory Sensory Neurons Detects Geosmin

We first set out to determine the behavioral significance of geosmin by using a T-maze (Figure 1B). In this two-choice olfactory assay, geosmin on its own elicited avoidance at very low concentrations (10^{-6}) (Figure 1C). For comparison, benzaldehyde—a well-known repellant to flies—in the same assay required a 1,000-fold higher dose than geosmin to trigger repulsion (Figure 1C). The actual fold difference in flies' behavioral sensitivity toward these two compounds is greater once volatility is factored in. The vapor pressure of geosmin is 1,000-fold lower than for benzaldehyde (0.001 mmHg versus 1.27 mmHg at 25°C). Thus, at a given dose and temperature, the number of geosmin molecules in vapor phase is substantially lower than for benzaldehyde. Geosmin is accordingly not only repellent but is also repellent when present in exceedingly low amounts.

Flies are evidently equipped with a sensitive detection system for geosmin. To identify the population of OSNs that is activated by geosmin, we next turned to electrophysiology. Specifically, we performed single-sensillum recording (SSR) measurements, a method that allowed us to assess odor-induced OSN activity extracellularly. We aimed to obtain SSR measurements from all antennal olfactory sensillum types while stimulating the contacted OSNs with geosmin. The ~450 olfactory sensilla of the fly antennae (Shanbhag et al., 1999) can be divided into 17 functional types, which in total house 46 functionally distinct OSN classes (de Bruyne et al., 2001; Hallem et al., 2004; Couto et al., 2005; Yao et al., 2005; van der Goes van Naters and Carlson, 2007; Benton et al., 2009). In addition to these well-classified sensilla, morphological data indicate that the antennae also contain one more type, the so-called intermediate sensilla; these sensilla house an unknown number of functional OSN classes (Shanbhag et al., 1999). The second olfactory organ of the fly, the maxillary palp, houses an additional three types for a total of six distinct OSN classes (de Bruyne et al., 1999). By performing a considerable number of SSR measurements ($n > 1000$) using diagnostic odors and by comparing the response properties of contacted OSNs with previously published ligand affinities, we were able to locate and record from all sensillum types present on the antennae (including two types of intermediate sensilla), as well as from the three types found on the maxillary palps (Figure 2A).

Response to geosmin came from just a single class of antennal OSNs, namely, the ab4B OSNs (Figures 2B and 2C). These neurons express the odorant receptors (OR) *Or56a* and *Or33a* (Couto et al., 2005; Fishilevich and Vosshall, 2005), of which only the former is functional in the *Canton-S* strain we used here (Kreher et al., 2008). Although ab4B OSNs have been measured from previously (e.g., de Bruyne et al., 2001), geosmin is the first ligand reported for this neuron class. To confirm that

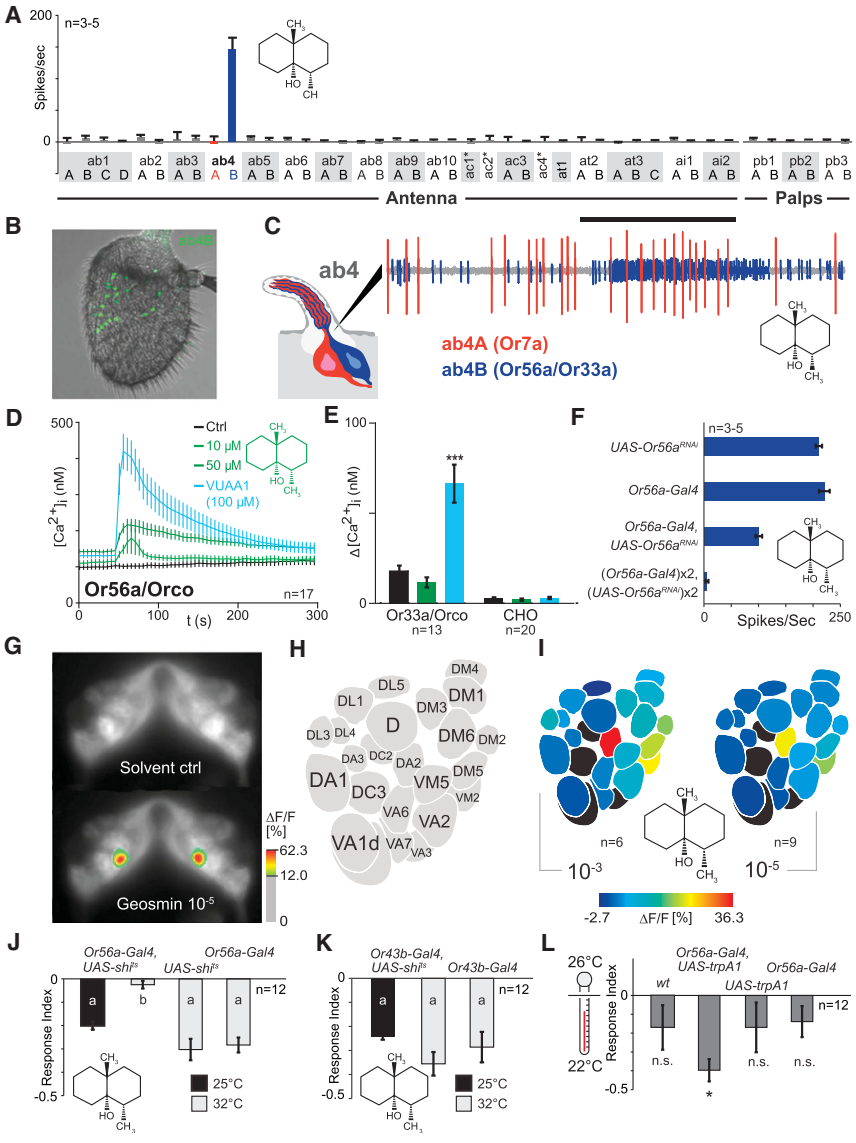

Figure 2. Geosmin Activates a Single Class of Antennal Olfactory Sensory Neurons

(A) SSR measurements from all olfactory sensilla with geosmin (10^{-3}) as a stimulus. ab, antennal basiconic sensilla (s.); ac, antennal coeloconic s.; at, antennal trichoid s.; ai, antennal intermediate s.; pb, palp basiconic s. Stars denote that activity from individual OSNs was not separated. Error bars represent SEM.

(B) Distribution of ab4B neurons on the antenna as visualized by the expression of GFP from the *Or56a* promoter.

(C) Representative SSR traces from an ab4 sensillum. The smaller amplitude spiking neuron, i.e., ab4B responds to geosmin (10^{-3}). The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(D) The free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in CHO cells expressing Or56a and Orco increases after the application of geosmin and VUAA1 (100 µM), but not of saline (control). Error bars represent SEM.

(E) Mean increase in free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in CHO cells expressing Orco and Or33a or nontransfected CHO cells after the application of saline (control), geosmin (50 µM), and VUAA1 (100 µM). Star denotes response significantly different from control (Student's t test, $p < 0.05$). Color scale as in (D). Error bars represent SEM.

(F) Quantification of responses to geosmin (10^{-3}) from ab4B OSNs of flies expressing RNAi against Or56a in the ab4B OSNs and the corresponding parental lines. Error bars represent SEM.

(G) False color-coded images showing solvent-induced (top) and geosmin-induced (bottom) calcium-dependent fluorescence changes in the AL of a fly expressing the activity reporter GCaMP3.0 from the *Orco* promoter.

(H) Glomerular atlas of the AL.

(I) Odor-induced activity plotted on schematic ALs (average % Δ*F*/*F*).

(J) RI to geosmin (10^{-5}) of flies expressing *Shibire^{ts}* from the *Or56a* promoter and corresponding parental lines in a T-maze assay. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(K) RIs to geosmin (10^{-5}) of flies expressing *Shibire^{ts}* from the *Or43b* promoter and the corresponding parental lines in a T-maze assay. No significant differences (ANOVA followed by Tukey's test; $p > 0.05$). Error bars represent SEM.

(L) RIs of flies expressing *dTRPA1* from the *Or56a* promoter, the corresponding parental lines, and WT in a T-maze assay confronted with a choice between 22 and 26°C. Deviation of the RI against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

See also Figure S1.

Or56a is indeed the geosmin receptor, we next expressed this protein in Chinese hamster ovary (CHO) cells that stably expressed the OR coreceptor *Orco* (Larsson et al., 2004). Because insect ORs are Ca^{2+}-permeable ionotropic receptors, OR activation can be monitored by measuring the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. The application of geosmin transiently increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 2D). The cells responding to geosmin were seen to respond to the Orco agonist VUAA1 (Jones et al., 2011), although there was no response to control application of saline (Figure 2D and Figure S1A available online). We then expressed *Or33a* in the same CHO cell line. Although the cells responded to VUAA1, we found no responses to geosmin (Figure 2E). CHO cells not expressing *Orco* or either of the two tuning ORs produced no Ca^{2+} signals in response to the application of geosmin or VUAA1 (Figure 2E). Loss of function of *Or56a* should render ab4B OSNs insensitive to geosmin. We next used SSR to examine the function of ab4B OSNs expressing a *UAS*-RNA interference (RNAi) construct against *Or56a*. The expression of *UAS-Or56a^{RNAi}* reduced the response to geosmin in a dose-dependent manner (Figures 2F and S1B). In flies carrying one copy each of *Or56a-Gal4* and *UAS-Or56a^{RNAi}*, the response to

geosmin was reduced by ~50% compared to the response displayed by the parental lineages. With two copies of each, the response was essentially abolished (~98% reduction) (Figure 2F). Thus, we conclude that *Or56a* alone underlies the ability of the ab4B cells to detect geosmin.

To further verify that geosmin is detected only by a single class of OSNs, we next employed functional imaging to examine the activity pattern in the antennal lobe (AL) evoked by geosmin (Figures 2G and S1C). We used the *Gal4-UAS* system to express the Ca^{2+}-sensitive reporter gene GCaMP3.0 (Tian et al., 2009) from the *Orco* promoter, thereby labeling all OSNs except those relying on ionotropic receptors (Benton et al., 2009) for odorant detection. Activated glomeruli were then identified by comparing the activation pattern with the map of the fly AL (Couto et al., 2005; Fishilevich and Vosshall, 2005) (Figure 2H). We stimulated flies with diagnostic odors to assist glomerular identification (data not shown) and with geosmin at 10^{-3} and 10^{-5} dilutions (Figures 2G and 2I). At 10^{-5}, geosmin elicited repeatable signals from only a single locus in the AL—the DA2 glomerulus, which receives input from ab4B neurons (Couto et al., 2005; Fishilevich and Vosshall, 2005). We note that DA2 is also situated in the same lateral part of the AL that has previously been implicated in handling aversive odors (Knaden et al., 2012). In a number of recordings, we also noted activity from VM2; however, these signals were not consistently reproducible. In the SSR screen, we never observed any activity in response to geosmin from OSNs innervating VM2; these OSNs are housed in the ab8 sensillum (Figure 2A). Hence, the activity noted from VM2 most likely does not reflect actual peripheral input but, rather, may stem from intrinsic AL processes. We therefore conclude that geosmin is indeed detected by a single class of OSNs. It should be stressed that the level of specificity shown here toward a nonpheromonal odor is most unusual, if not unique, among the olfactory systems investigated to date.

Activation of the ab4B Neurons Is Necessary and Sufficient for the Aversive Behavior

If the behavior triggered by geosmin is solely derived from the activity of ab4B neurons, silencing this OSN subpopulation should also abolish the aversive behavior. To silence these neurons, we expressed the temperature-sensitive mutant dynamin *Shibire^{ts}* (Kitamoto, 2001) from the *Or56a* promoter. At the restrictive temperature (32°C), flies carrying this construct displayed no aversive behavior toward geosmin (Figure 2J). The same flies, tested at a permissive temperature (25°C), showed a strong aversion to geosmin. Parental lines tested at the nonpermissive temperature showed a somewhat increased repellency, which was likely caused by the increased volatility of geosmin at the higher temperature. Silencing the ab4B neurons had no effect on flies' behavior in response to benzaldehyde (Figure S1D). In line with the SSR experiments, silencing input to VM2—via the expression of *Shibire^{ts}* from the *Or43b* promoter—did not affect flies' behavior in response to geosmin (Figure 2K). The ab4B OSNs are evidently necessary for the aversive behavior.

We next asked whether selectively activating these neurons is sufficient to cause aversion. We expressed the temperature-sensitive cation channel *dTRPA1* in the ab4B neurons, a procedure that allowed us to conditionally activate these OSNs at temperatures >26°C (Hamada et al., 2008). As a control, we first examined the temperature preference (26°C versus 22°C) of wild-type (WT) flies in a T-maze assay. WT flies showed a tendency toward aversion against the higher temperature (Figure 2L). Having established baseline behavior in the assay, we next asked whether flies bearing the *Or56a-Gal4*, *UAS-dTRPA1* construct displayed a stronger aversion toward the higher temperature. In fact, flies expressing *dTRPA1* in ab4B OSNs showed significant avoidance toward the warm side, whereas parental control flies showed moderate (but insignificant) aversion (Figure 2L). Thus, specifically activating these neurons induces aversion in flies. In summary, these experiments demonstrate that the aversive behavior caused by geosmin is mediated solely through a single class of OSNs.

The ab4B Neurons Respond Exclusively to Geosmin

As seen, geosmin is detected by a single class of OSNs, ab4B. We next asked whether or not these neurons are exclusively tuned to geosmin. We again used SSR but now screened with 103 structurally diverse odorants (tested at 10^{-2} dilution) (Figure S2A). The larger spiking neuron in the ab4 sensillum responded to a range of compounds (Figure S2B). Interestingly, we note that the most potent ligands for these OSNs are all known repellants. The functional significance, if any, of having two neurons both responding to aversive odorants that are cocompartmentalized is unclear. The ab4B neurons, in contrast, displayed a striking degree of selectivity, as none of the screened odorants—apart from geosmin—elicited any increased spike firing (Figure 3A). Showing specificity in the context of the olfactory system is, however, difficult, as there are thousands of volatile chemicals in nature. Our tested set thus represents only a fraction of the volatile chemicals potentially present in the natural habitat of *D. melanogaster*.

To address this issue and to more firmly examine the specificity of these neurons, we next expanded our SSR investigation by using a gas chromatograph (GC) for stimulus delivery. GC-linked SSR enables the screening of headspace collections from complex odor sources and, consequently, enables the probing of large numbers of volatiles. We first sampled odors from a wide range of sources present in the natural habitat of *D. melanogaster* in native Africa as well as in the "Diaspora." We collected odors from 14 sources, including avoided ones, such as feces (from African mammals) and rotting meat, as well as attractive ones, such as fruits and vinegar. The total number of volatiles present in these samples is difficult to firmly establish, but the number of distinguishable flame ionization detection (FID) peaks amounts to ~2,900 in total. The actual number of compounds present is, however, likely considerably higher. The headspace of many fruits typically contains >400 volatiles (e.g., Petro-Turza, 1987); hence, in our samples, many more compounds were presumably present but only in amounts below the FID limit. These compounds were nevertheless effectively screened, as insects, including *Drosophila*, are capable of detecting compounds present well below the FID limit.

Having collected and verified the odor samples, we then proceeded to perform GC-SSR measurements from ab4B neurons.

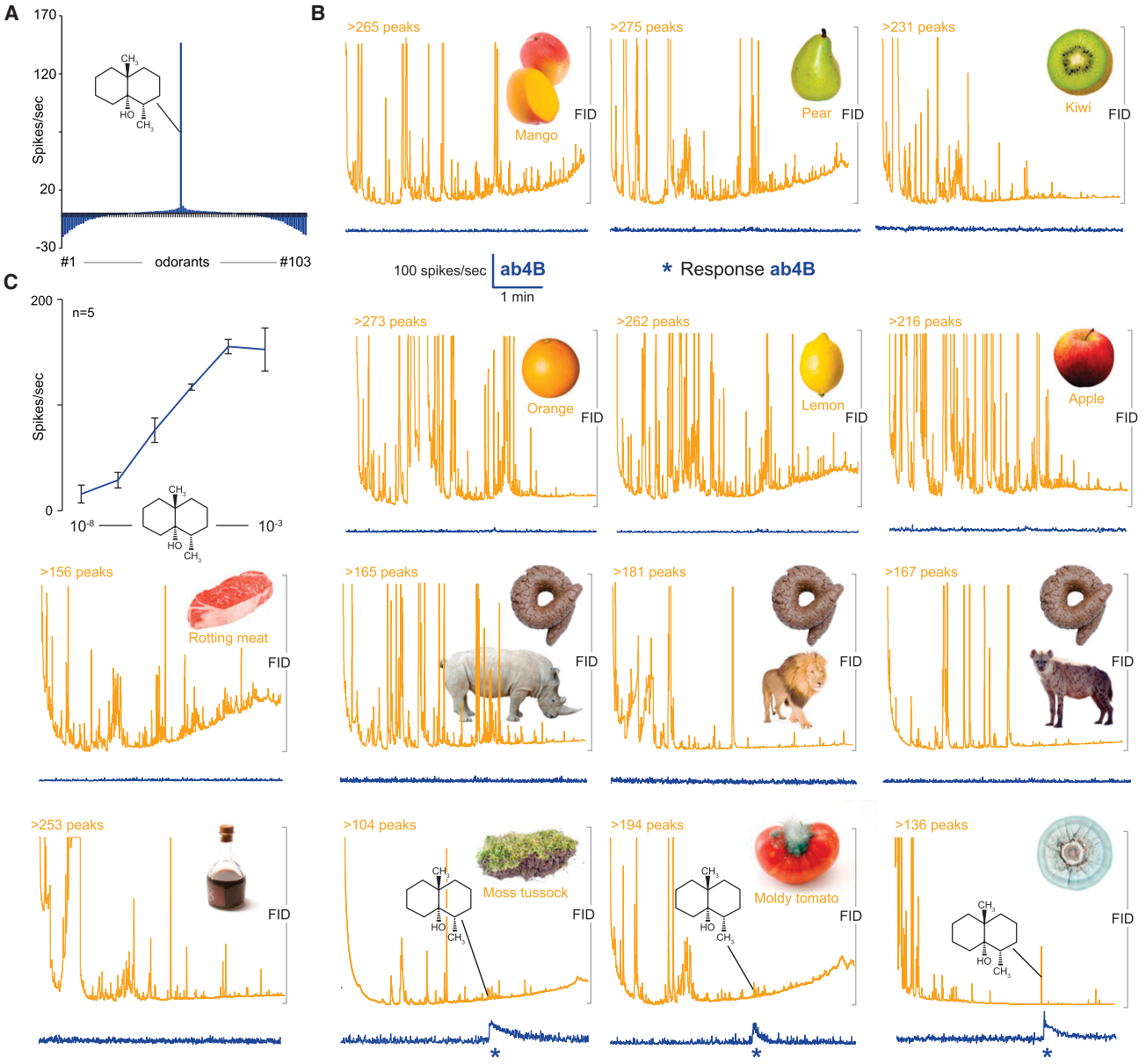

Figure 3. The ab4B Neurons Respond Exclusively to Geosmin

(A) Tuning curve for the ab4B neuron type based on a screen of 103 synthetic substances (10^{-2} dilution). Error bars represent SEM.

(B) Gas-chromatography-linked SSR measurements from ab4B neurons. The orange trace represents the FID, photos depict the screened odor sources, and the blue trace depicts the simultaneously recorded neural activity of ab4B neurons. Stars denote response. n = 1–3.

(C) Dose response curve from ab4B neurons toward geosmin. Error bars represent SEM.

See also Figure S2.

Out of the 14 odor samples we screened, only three evoked responses (Figure 3B), namely the headspace of a moldy tomato, a moss tussock, and isolated cultures of the common soil bacterium *Streptomyces coelicolor*. In each of the active samples, only a single FID peak elicited a response. We next used GC-linked mass spectroscopy (GC-MS) combined with synthetic standards to identify the functionally relevant peaks in these three samples; in all cases, these turned out to be geosmin. Thus, the ab4B neurons are indeed extremely specific, and it is reasonable to conclude that the sole function of these neurons is to detect geosmin.

How sensitive are the ab4B neurons toward geosmin? Our T-maze experiments (Figure 1C) had already shown that the flies respond behaviorally at very low concentrations. Indeed, the ab4B neurons respond to geosmin at 10^{-8} dilution (corresponding to 100 pg of substance in the stimulus pipette)

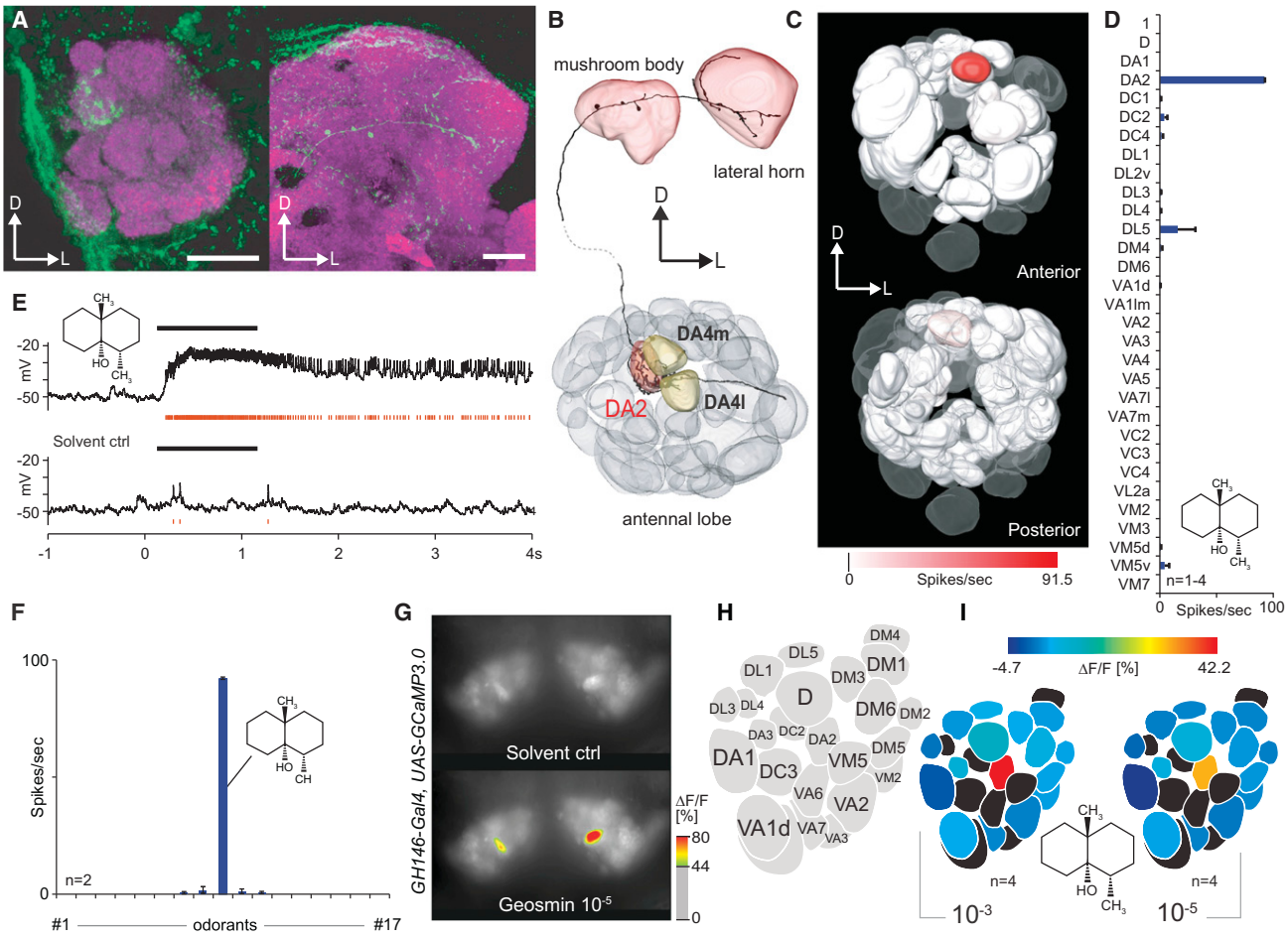

Figure 4. Geosmin Activates a Functionally Segregated Pathway

(A) A PN innervating the DA2 glomerulus (left) and sending its axon to the calyx of the mushroom body and terminating in the lateral horn (right). PN, green; nc82, magenta. D denotes dorsal, and L denotes lateral.

(B) Reconstruction of the neuron in (A).

(C) Glomeruli from which PN recordings were obtained (in solid), with the response to geosmin (10^{-3}) false color coded. Transparent glomeruli were not investigated.

(D) The net change in spike frequency in response to geosmin (10^{-3}) stimulation from PNs innervating 31 glomeruli. Error bars represent SEM.

(E) Example spike trace from a DA2 PN responding to geosmin (10^{-3}). Black bar marks the 1 s odor stimulus. Red trace represents extracted spikes.

(F) Tuning curve for DA2 PNs based on 17 synthetic substances (10^{-2} dilution, except geosmin, which was used at 10^{-3}). Error bars represent SEM.

(G) False color-coded images showing solvent-induced (top) and geosmin-induced (bottom) calcium-dependent fluorescence changes in AL PNs of a fly bearing the *GH146-Gal4*, *UAS-GCaMP3.0* constructs.

(H) Glomerular atlas of the AL.

(I) Odor-induced activity plotted on schematic ALs (average % $\Delta F/F$).

See also Figure S3.

(Figure 3C), which is in good agreement with the dilution of geosmin (1.74×10^{-7}) causing reduced upwind flight attraction to vinegar headspace when vaporized in the wind tunnel (Becher et al., 2010).

Geosmin Triggers a Segregated Pathway through the Antennal Lobe to Higher Brain Centers

How is the specific tuning in flies to geosmin seen in the peripheral sensory neurons transferred to higher brain centers? In *Drosophila*, the OSNs form synapses with projection neurons (PNs) and local interneurons within the AL. Most PNs innervate only a single glomerulus (Figures 4A and 4B), whereas local interneurons typically show broad innervation throughout the AL. The PNs send their axons to the mushroom body and lateral horn (Figures 4A and 4B) (Vosshall and Stocker, 2007). PNs tend to respond to a somewhat broader range of odors than do their corresponding OSNs (Wilson et al., 2004; Bhandawat et al., 2007). For instance, the PNs connected to OSNs that respond only to geranyl acetate respond to additional odors as well. However, PNs connected to OSNs that respond to the sex

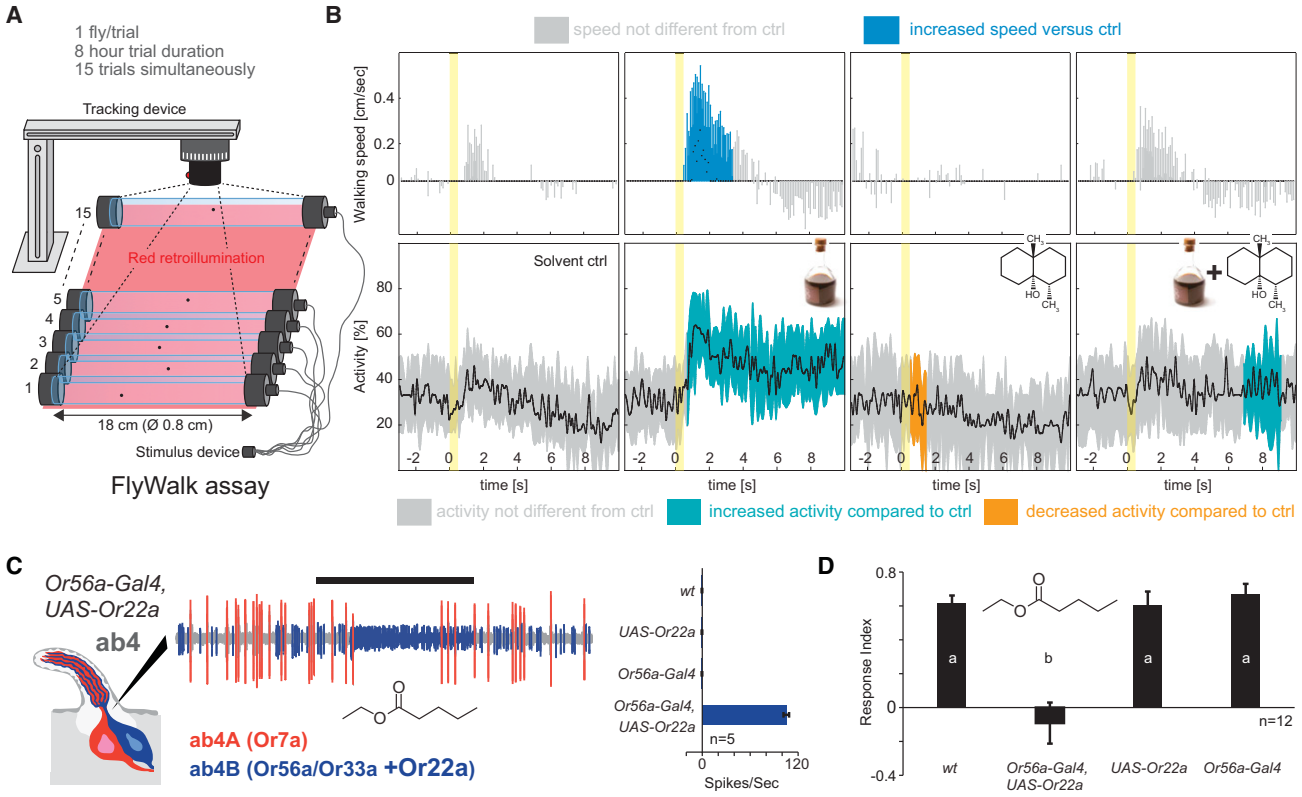

Figure 5. Activation of the Geosmin Pathway Reduces Attraction

(A) Schematic drawing of the Flywalk assay used in (B). For details, see Steck et al. (2012).

(B) Quantified behavior from individual flies stimulated with balsamic vinegar, geosmin (10^{-3}), and a mix of the two in the Flywalk assay. Top graphs, box plot representations of odor-induced changes in upwind speed of flies (n = 30); black line represents median upwind speed; box, interquartile range; whiskers, 90th and 10th percentiles. Lower graphs, undirected activity of flies (n = 30); black line, median activity; shaded area, interquartile range. Yellow area marks the 500 ms odor stimulus. Statistical analysis per Steck et al. (2012).

(C) Left, representative SSR trace from an ab4 sensillum, stimulated with ethyl butyrate (10^{-5}) in which the B neuron expresses Or22a. Right, quantification of mean responses to ethyl butyrate from control ab4B OSNs and ab4B OSNs misexpressing Or22a.

(D) Response indices of flies expressing Or22a in the ab4B OSNs, corresponding parental lines and WT flies to ethyl butyrate (10^{-5}) in a T-maze assay. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

See also Figure S4.

pheromone cVA do not show a broad response pattern and are just as specific as their cognate OSNs (Schlief and Wilson, 2007). We thus asked: how specific is the response of PNs that respond to geosmin?

We carried out whole-cell patch-clamp recordings from a large number of randomly selected uniglomerular PNs, stimulating with 17 chemicals, including geosmin (Figure S3). We obtained recordings and fills from 66 PNs (from 66 individual flies), which covered 31 different glomeruli. Geosmin elicited significant responses only from two PNs, both of which innervated the DA2 glomerulus (Figures 4A–4E). Although not all glomeruli were covered, this result strongly suggests that geosmin information does not diffuse broadly across the AL to other glomeruli. Moreover, DA2 PNs appear to be as selective as the input OSNs because these PNs responded exclusively to geosmin and not to any of the other screened compounds (Figures 4F and S3). To further examine the specificity of the AL output, we next imaged flies carrying the *GH146-Gal4* and *UAS-GCaMP3.0* constructs in which ~1/2 of the PNs express the GCaMP3.0 activity reporter (Stocker et al., 1997; Jefferis et al., 2001). Stimulation with geosmin again exclusively activated the DA2 glomerulus (Figures 4G–4I). Thus, we conclude that, like the labeled line pheromone pathway, the geosmin circuit forms a dedicated functionally segregated pathway, at least to the point of the calyx and lateral horn. The fate of the signal past this point remains to be elucidated.

The Geosmin Circuitry Can Modulate and Override Innate Attraction

As mentioned before, the addition of geosmin to vinegar significantly reduced positive chemotaxis in flies' response to this innately attractive odor. To verify that geosmin indeed has the capacity to reduce flies' attraction to vinegar, we next repeated the wind tunnel experiments with an alternative bioassay, the Flywalk (Steck et al., 2012) (Figure 5A). This assay enables high-resolution quantification of behavior from individual flies in

response to short pulses of an odor stimulus repeated during an extended period of time. Our Flywalk results parallel the findings from the wind tunnel (Figure 5B). Exposing flies to pulses of balsamic vinegar induced bursts of positive chemotaxis, which were significantly reduced when geosmin was added to the vinegar volatiles. Geosmin alone induced a "freezing" behavior, i.e., a decrease of the flies' activity, which, in this assay, reflects aversion (Steck et al., 2012). The ability of geosmin to reduce the attractiveness of vinegar is robust and can be repeated with both the trap assay (Larsson et al., 2004) (Figures S4A and S4B) and the T-maze (Figure S4C).

In light of the physiology findings, the cause of the reduced attractiveness of the geosmin-vinegar mix should stem from activation of the DA2 pathway. This circuit should consequently have the capacity to override and modulate an innate behavior. To test this notion, we used the *Or56a-Gal4* line to drive the expression of an additional odorant receptor (*Or22a* targeting glomerulus DM2) in ab4B OSNs (Figure 5C), enabling us to manipulate the activity of the DA2 circuit in the absence of geosmin and thereby to separate the chemical from the actual effect. In flies expressing Or22a under the *Or56a* promoter, stimulation with ethyl butyrate, a potent ligand for Or22a that is highly attractive to flies (Figure 5D), should result in the activation of both DM2 and DA2, in turn reducing the flies' attraction to ethyl butyrate. Through SSR, we first verified that the misexpression of Or22a conferred sensitivity toward ethyl butyrate in ab4B neurons (Figure 5C). Having established physiological function, we then tested the flies' behavioral response toward ethyl butyrate by using a T-maze. The parental control lines showed the expected strong positive response of WT flies toward this fruit ester. On the other hand, flies additionally expressing Or22a in the ab4B OSNs showed no attraction toward ethyl butyrate (Figure 5D). Thus, activating DA2 and the associated pathway can modulate and override innate attractive behavior.

Geosmin Is Used by the Fly to Detect Toxic Molds and Bacteria

We next asked what the possible evolutionary and ecological reason might be for the strong and hard-wired chemosensory avoidance of geosmin. Because geosmin itself is nontoxic to invertebrates as well as mammals (Young et al., 1996), the function of the circuit is not just to alert *D. melanogaster* to the presence of this compound. With some exceptions, the majority of volatiles flies detect are widely produced in nature and, thus, are difficult to firmly associate with a specific source. Geosmin—although very abundant in nature—is solely produced by a narrow range of microbes, in particular *Penicillium* fungal molds (Mattheis and Roberts, 1992) and *Streptomyces* soil bacteria (Gerber and Lechevalier, 1965). Has the system for detecting geosmin evolved to identify these specific microorganisms? We first examined whether flies could survive on these types of microbes. We transferred newly eclosed flies to vials with a yeast-containing medium or to vials additionally containing cultures of either *Streptomyces coelicolor* or *Penicillium expansum*. Flies were unable to survive in the presence of either of these microbes (Figure 6A), presumably due to the accumulation of toxins. Many fungal molds, including *P. expansum*, produce a range of toxic secondary metabolites, several of which have been shown to have strong insecticidal activity (Castillo et al., 1999). Many geosmin-producing microbes are not only toxic but are also known to outcompete or even kill the yeasts flies graze on (Arndt et al., 1999). Thus, for the fly, being able to detect and avoid fruit colonized by harmful molds and bacteria should be an essential skill.

Because many geosmin-producing microbes are detrimental to flies, we suspected that substrates colonized by this type of microbe are avoided for oviposition. Thus, we next looked for an olfactory-based oviposition preference in flies by using a two-choice assay (Figure 6B) in which flies were given the option of laying eggs on plates containing either standard *Drosophila* yeast medium or on plates additionally inoculated with *S. coelicolor*. Indeed, flies avoided laying eggs on plates containing *S. coelicolor* (Figure 6C). Is the avoidance of the bacterial plates mediated via geosmin? To address this question, we subsequently repeated the oviposition experiments. We inoculated one of the plates with a gene-targeted *S. coelicolor* strain (J3001), which carries a deletion in a key gene involved in the geosmin synthesis pathway (Gust et al., 2003). The J3001 strain is thus identical to WT *S. coelicolor* except for its inability to produce geosmin, the lack of which we also confirmed via GC-MS and GC-SSR (Figure 6D). Abolishing the production of geosmin completely eliminated the avoidance in response to *S. coelicolor* (Figure 6C). In the absence of geosmin, flies readily oviposited on the harmful media. Eggs deposited onto *S. coelicolor* did not develop into adult flies (data not shown), and survival on the J3001 strain did not differ from survival on WT *S. coelicolor* (log rank test; $p = 0.22$). In a pure olfactory choice assay, the trap assay (Figure S4A), flies also discriminated between the two strains, preferring J3001 over WT (Figure S5).

We next wondered whether the reluctance to oviposit in the presence of (WT) *S. coelicolor* is dependent on the DA2 circuit. To address this question, we examined the oviposition preference of flies carrying the previously used *Or56a-Gal4*, *UAS-Shibire^{ts}* construct. At permissive temperatures, these flies strongly avoided plates containing *S. coelicolor*, whereas at restrictive temperatures, there was no avoidance, and the flies even showed a slight preference for the bacterial substrate (Figure 6E). In line with our hypothesis, the presence of geosmin alone should also prevent egg laying, which it did. Plates containing geosmin (10^{-3}) were avoided as an oviposition substrate (Figure 6F). One could speculate that the presence of any strongly repellent odor would also prevent oviposition from occurring. However, benzaldehyde did not inhibit oviposition from occurring at 10^{-4} and 10^{-2} dilutions and barely did so even when tested as a pure substance (Figure 6F).

Are flies also hesitant to consume food contaminated with this type of microbe? We next examined feeding preference by using a capillary feeder assay (Figure 6G) (Ja et al., 2007); here, flies could choose between two 5% sucrose solutions, one of which was based on a wash from WT *S. coelicolor* colonies. Indeed, flies clearly preferred the pure sucrose solution (Figure 6H). We then repeated these experiments, replacing the WT *S. coelicolor* with the J3001 strain. The solution

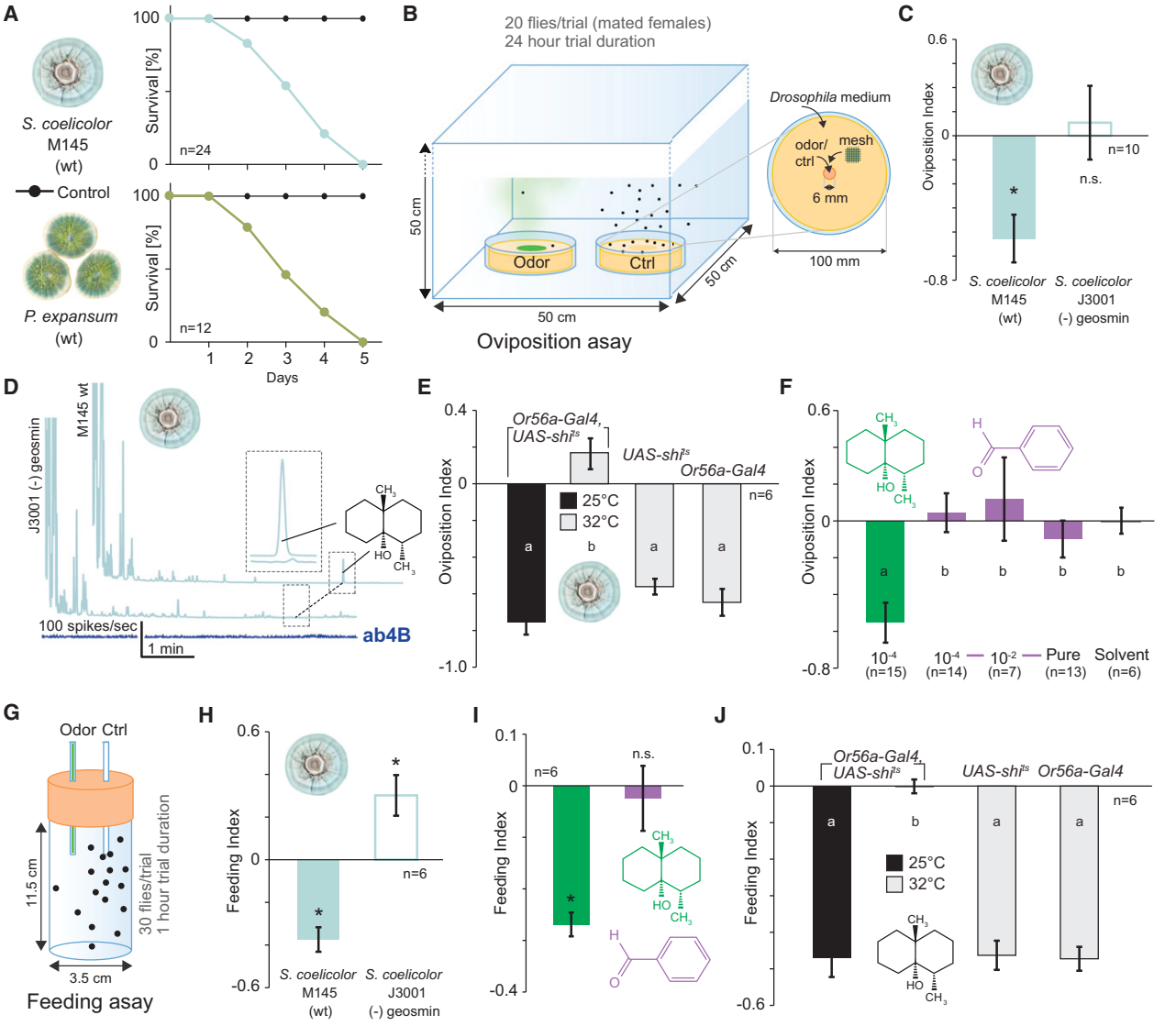

Figure 6. Geosmin Is Used by Flies to Detect Toxic Molds and Bacteria

(A) Survival rate of newly eclosed flies transferred to vials containing pure agar medium or medium with 1-week-old cultures of either of two geosmin-producing microbes.

(B) Schematic drawing of the oviposition choice assay used in (C), (E), and (F).

(C) Oviposition indices (OI) to WT (M145) and J3001 *S. coelicolor* of WT flies. The J3001 only differs from WT by its inability to produce geosmin. Deviation of the oviposition index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(D) GC-MS and GC-SSR analysis of headspace from J3001 and M145. Pale blue represents flame ionization detection traces. The dark blue trace shows activity from an ab4B OSN being stimulated with J3001 headspace (no response).

(E) OIs to WT *S. coelicolor* of flies expressing *Shibire*^{ts} in the ab4B OSNs and corresponding parental lines at permissive (25°C) and restrictive (32°C) temperatures. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(F) OIs to geosmin and benzaldehyde of WT flies. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(G) Schematic drawing of the capillary feeding assay (modified from Ja et al. [2007]) used in (H)–(J).

(H) Feeding indices (FI) to 5% sucrose solutions containing traces of WT (M145) or J3001 *S. coelicolor* of WT flies. Deviation of the feeding index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(I) FIs to 5% sucrose solutions containing geosmin (0.1%) or benzaldehyde (0.1%) of WT flies. Deviation of the feeding index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(J) FIs to 5% sucrose solutions containing traces of WT (M145) *S. coelicolor* of flies expressing *Shibire*^{ts} from the *Or56a* promoter and corresponding parental lines at permissive (25°C) and restrictive (32°C) temperatures. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

See also Figure S5.

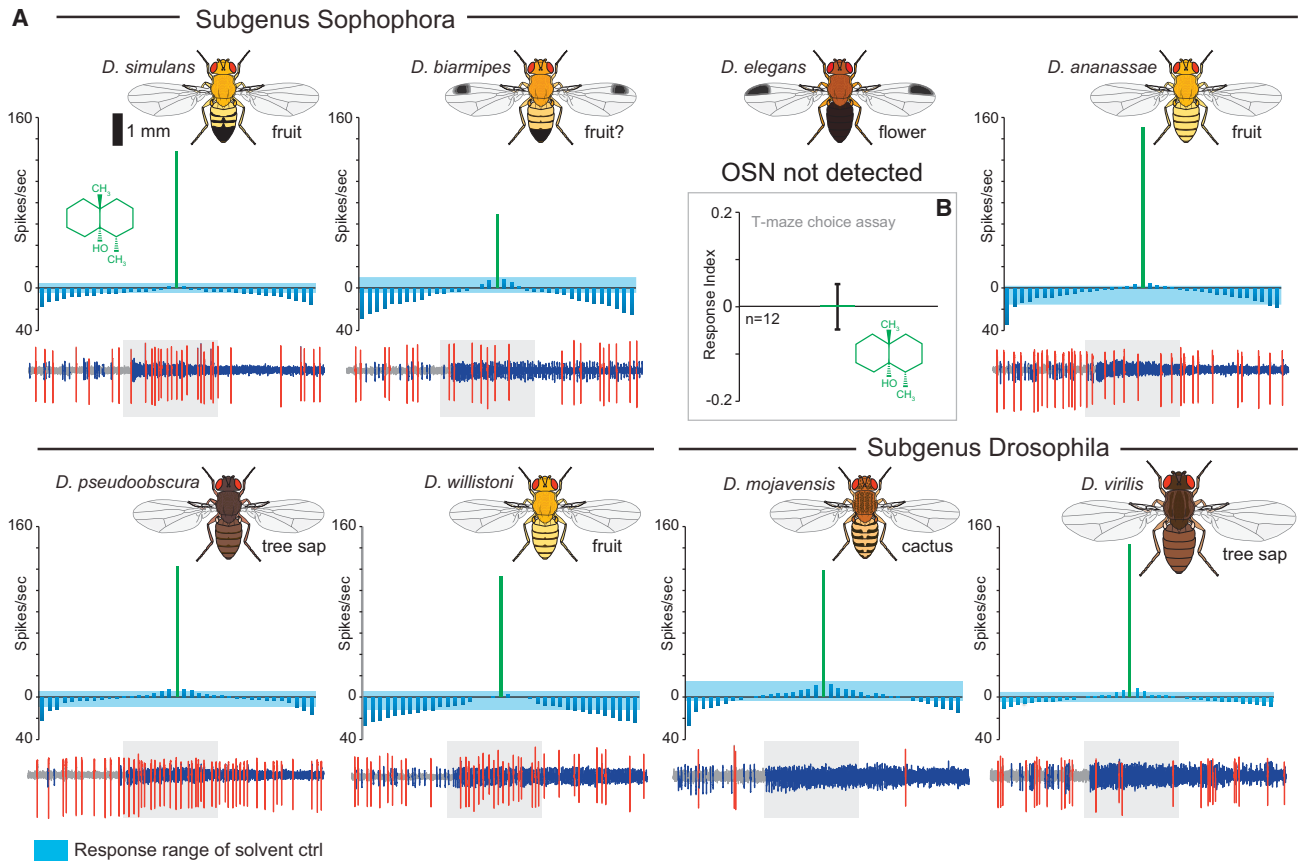

Figure 7. Responses to Geosmin in Drosophilids Are Deeply Conserved

(A) Tuning curves for neurons with similar response properties to the ab4B neurons of *D. melanogaster* from select members of the genus *Drosophila* (n = 3 for all species). The tuning curves are based on a screen with 37 compounds, tested at 10^{-2}. Below curves are representative SSR traces showing responses to geosmin (10^{-3}), with the gray box indicating the 0.5 s stimulus delivery period. The natural breeding substrates are indicated underneath the schematic drawings of the species. Error bars represent SEM.

(B) Response index to geosmin (10^{-5}) of *D. elegans* in a T-maze assay. Deviation of the response index against zero was tested with a Student's t test (not significant). Error bars represent SEM.

See also Figure S6.

containing J3001 did not reduce feeding but was slightly preferred over the sucrose-only solution (Figure 6H), suggesting that the aversion is due to the presence of geosmin. In line with this observation, adding geosmin (0.1%) also reduced feeding (Figure 6I). The addition of another aversive odor, benzaldehyde (0.1%), had no effect on feeding (Figure 6I). We next wondered whether the feeding aversion is due to olfactory input to the DA2 pathway. Indeed, the reduced feeding stems not from geosmin having an aversive taste but from the activation of ab4B OSNs because silencing input to this pathway—via *Shibire^{ts}*—also fully abolished the geosmin-induced feeding aversion (Figure 6J). Thus, geosmin also functions as an antifeedant, operating via the olfactory system.

Taken together, these findings strongly suggest that the ecological significance of geosmin is to alert flies to the presence of toxic molds and bacteria. The geosmin circuit performs a critical task, providing flies with a reliable and sensitive means of identifying unsuitable hosts.

The Geosmin Detection System Is Conserved across the Genus *Drosophila*

To shed light on the origin and evolution of the geosmin detection system circuit, we next turned to a comparative approach. We tested eight drosophilid species—chosen based on genome availability and phylogenetic and ecological considerations—for their capacity to detect geosmin (Figure S6A). We set out to identify neurons able to detect geosmin via SSR, stimulating with a set of 37 chemically diverse odorants (at 10^{-2} dilution) (Figure S3D). We located OSNs tuned to geosmin in all the screened species except *D. elegans* (Figure 7A). Electroantennogram recordings from this species also showed no response to geosmin (data not shown) and neither does this species respond behaviorally to geosmin in a T-maze assay (Figure 7B). As in *D. melanogaster*, in each of the species responding to geosmin, detection was noted only from a single class of OSNs, which also responded exclusively to geosmin (Figure 7A). The geosmin OSNs we found in the other species may well

serve the same function that they serve in *D. melanogaster*. The lack of a geosmin detection system in *D. elegans* may be a consequence of the low susceptibility to mold growth of this species' breeding substrate, namely, fresh flowers (Yoshida et al., 2000). Putatively functional orthologs of *Or56a* are also present across the species in which we have complete OR repertoires (Guo and Kim, 2007). We also located intact orthologs of *Or56a* in draft genome assemblies from an additional eight drosophilids (Figure S6B), including *D. biarmipes* and *D. elegans*. The function (if any) of the *Or56a* ortholog in the latter remains unknown. Analysis of selection pressure also showed that the *Or56a* genes are under overall purifying selection (Figure S6C). The response properties of the second neuron residing in these sensilla are much less conserved (Figure S6D). These neurons also do not express orthologous receptors across the examined species. In *D. melanogaster*, the ab4A neurons express Or7a (Hallem et al., 2004), orthologs of which are, however, found only in the subgenus *Sophophora* (Guo and Kim, 2007). Yet, also in species in which we can assume that Or7a underlies the response property, we did note variation in ligand affinity. The function of the ab4A OSNs hence likely reflects species-specific requirements. The striking specificity toward geosmin seen in the olfactory system of *D. melanogaster* is accordingly a basal feature of the genus *Drosophila*, conserved for at least ~40 million years (Russo et al., 1995).

Conclusions

The manner in which flies decode and rely upon geosmin has few, if any, direct parallels. Comparable circuits are essentially found only within the subset of the olfactory nervous system that relays pheromone information. However, also within this context, it is exceedingly rare for animals to rely on just a single chemical to identify a critical resource. Almost all pheromones characterized to date have been complex blends processed by multiple neuronal pathways. Moreover, the specificity toward geosmin shown here surpasses many pheromone-tuned neurons; if presented with enough odorants or with odorants in sufficient concentration, these neurons will also display responses to other substances (Hansson and Stensmyr, 2011).

The closest match to the geosmin pathway is found outside of the regular olfactory system, namely in the detection and processing machinery for the atmospheric trace gas CO_2. Although CO_2 is a fundamentally different chemical from geosmin, the similarity in which these two stimuli are decoded is striking. In flies, the CO_2 circuit forms a functionally segregated pathway that mediates innate avoidance. Input to the CO_2 circuit is likewise fed by sensory neurons exclusively tuned to a single stimulus (Suh et al., 2004). Although organized similarly, the ecological significance of these two circuits seems to differ. Geosmin is used by flies as a universal warning sign for the presence of toxic compounds that are comorbid with geosmin. The evolutionary significance of this circuit is clear: it provides flies with a sensitive and specific means to identify unsuitable hosts. The ecological meaning of CO_2 for *D. melanogaster* is, however, unclear. In fact, it is puzzling why flies would be repelled by CO_2 at all. *D. melanogaster* is highly adapted toward breeding (and feeding) on substrates with high ethanol content. Because CO_2 is a ubiquitous byproduct of alcoholic fermentation, it would make an ideal cue for flies to follow when searching for suitable hosts. Elucidating the role of CO_2 from the point of view of flies and using assays that better reflect the natural setting should be a focus of future studies.

Circuits analogous to the geosmin pathway are a likely feature in the olfactory systems of most, if not all, insects. Although these circuits are probably similar mechanistically and functionally (i.e., selective with regards to input, mediating innate aversion, and abolishing attraction), the identity of the eliciting stimulus will differ, reflecting the demands raised by the taxon-specific ecology.

EXPERIMENTAL PROCEDURES

Fly Stocks

All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the *Drosophila* species stock center (https://stockcenter.ucsd.edu/info/welcome.php). Transgenic lines were obtained from the Bloomington *Drosophila* stock center (http://flystocks.bio.indiana.edu/), except for *UAS-Or22a*, which was donated by L. Vosshall (The Rockefeller University, New York) and *UAS-Or56-a^{RNAi}*, which was obtained from the Vienna RNAi stock center (http://www.vdrc.at).

Stimuli and Chemical Analysis

All synthetic odorants tested were acquired from commercial sources (Sigma, http://www.sigma-aldrich.com and Bedoukian, http://www.bedoukian.com) and were of the highest purity available. (±)-Geosmin (of >97% purity) was obtained from Sigma. Stimuli preparation and delivery followed Stökl et al. (2010). The headspace collection of volatiles was carried out according to standard procedures. *S. coelicolor* M145 and J3001 strains were gifts from K. Flärdh (Lund University, Sweden) and K. Chater (John Innes Centre, UK), respectively. *P. expansum* was obtained from Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl). Microorganisms were kept on strain-specific media (HiMedia, http://www.himedialabs.com), following standard protocols. Mammalian fecal samples were provided by the Leipzig Zoo. For GC stimulation, 1 μl of the odor sample was injected onto a DB5 column (Agilent Technologies, http://www.agilent.com), fitted in an Agilent 6890 GC, equipped with a four-arm effluent splitter (Gerstel, www.gerstel.com), and operated as previously described (Stökl et al., 2010) except for the temperature increase, which was set at 15°C min^{-1}. GC-separated components were introduced into a humidified airstream (200 ml min^{-1}) directed toward the antennae of a mounted fly. Signals from OSNs and FID were recorded simultaneously. GC-MS analysis was performed as previously described (Stökl et al., 2010).

Behavioral Assays

T-maze experiments were conducted as shown in Figure 1B, with flies starved for 4 hr prior to experiments with water provided ad libitum. The response index (RI) was calculated as (O-C)/T, where O is the number of flies in the baited arm, C is the number of flies in the control arm, and T is the total number of flies used in the trial. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). Trap assay experiments (Figure S4A) were performed as described in Stökl et al. (2010) with RI calculated as above. The Flywalk experiments followed protocols outlined in Steck et al. (2012) (Figure 5A). Survival was measured for individual flies (males and females, except for tests with J3001, in which only females were examined), which were kept for 5 days (at 23°C) in glass tubes (16 × 100 mm) with metal caps containing 1-week-old cultures of *S. coelicolor* or *P. expansum* grown on yeast-containing media (HiMedia). Oviposition experiments were carried out as shown in Figure 6B. Oviposition index was calculated as (O-C)/(O+C), where O is the number of eggs on a baited plate, and C is the number of

eggs on a control plate. Feeding experiments were conducted as described in Figure 6G. A feeding index was calculated as (O-C)/(O+C), where O is the amount of food consumed from odorous solutions, and C is the amount from control sucrose-only solutions.

Physiology and Morphology

Electroantennogram (EAG) recordings were performed following standard procedures (e.g., Stökl et al., 2010). For SSR measurements, the recording electrode and the reference electrode (inserted into the eye) were positioned under a microscope (Olympus BX51W1; http://www.olympus.com). The recording electrode was positioned by using a motorized, piezo-translator-equipped micromanipulator (Märzhauser DC-3K/PM-10; http://www.marzhauser.com/de/). The signal was amplified (Syntech UN-06, http://www.syntech.nl), digitally converted (Syntech IDAC-4), and finally visualized and analyzed by using Syntech AutoSpike v3.2. CHO cells stably expressing dOrco (Trenzyme, http://www.trenzyme.com) were transiently transfected with dOr56a/pcDNA3.1(−) or dOr33a/pcDNA3.1(−) by using a Roti-Fect transfection kit (Carl Roth, http://www.carlroth.com) as described (Sargsyan et al., 2011). Ca^{2+} imaging of CHO cells was performed as described (Wicher et al., 2008). The functional imaging of odor-induced glomerular activity was conducted as outlined in Stökl et al. (2010). Patch-clamp recording was performed as previously described (Seki et al., 2010), except that in vivo preparation was used, and odor stimuli were given. Preparation followed Stökl et al. (2010), with the exception that the neurolemma was removed to allow the recording electrode access to the cell bodies of the PNs. Spike analysis, immunohistochemistry, laser scanning microscopy, and 3D reconstructions were performed as previously described (Seki et al., 2010).

Statistics and Bioinformatics

Estimates of the selection pressure were done by maximum likelihood as implemented in PAML (Yang, 1997). Additional orthologs of *Or56a* were identified via TBLASTN searches of draft genomes (courtesy of modENCODE/Baylor College of Medicine), downloaded from http://www.ncbi.nlm.nih.gov/bioproject/63477.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.09.046.

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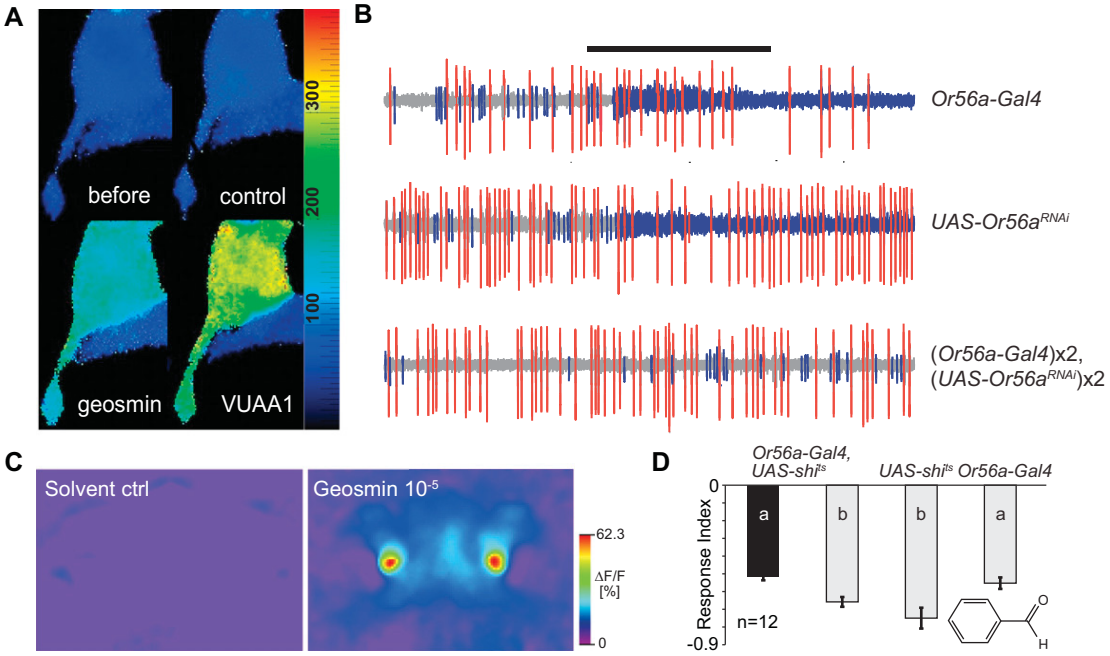

Figure S1. Molecular Function of Or56a, Related to Figure 2

(A) Color coded $[Ca^{2+}]_i$ (scaling bar, nM) in a CHO cell expressing *Or56a* and *Orco* before and 10 s after application of saline (control), geosmin (50 μM) and VUAA1 (100 μM).

(B) Representative SSR traces from control ab4 sensilla (top two traces) and from an ab4 sensillum with reduced levels of Or56a (bottom trace). Expression of RNAi directed against Or56a in ab4B OSNs (blue spikes) abolishes the response to geosmin (10^{-3}). Duration of the stimulus delivery (0.5 s) is marked by the black bar.

(C) Raw images from the same recording as in Figure 2G.

(D) Silencing ab4B neurons, via *Shibire^{ts}*, does not abolish aversion toward benzaldehyde (10^{-2} dilution). Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

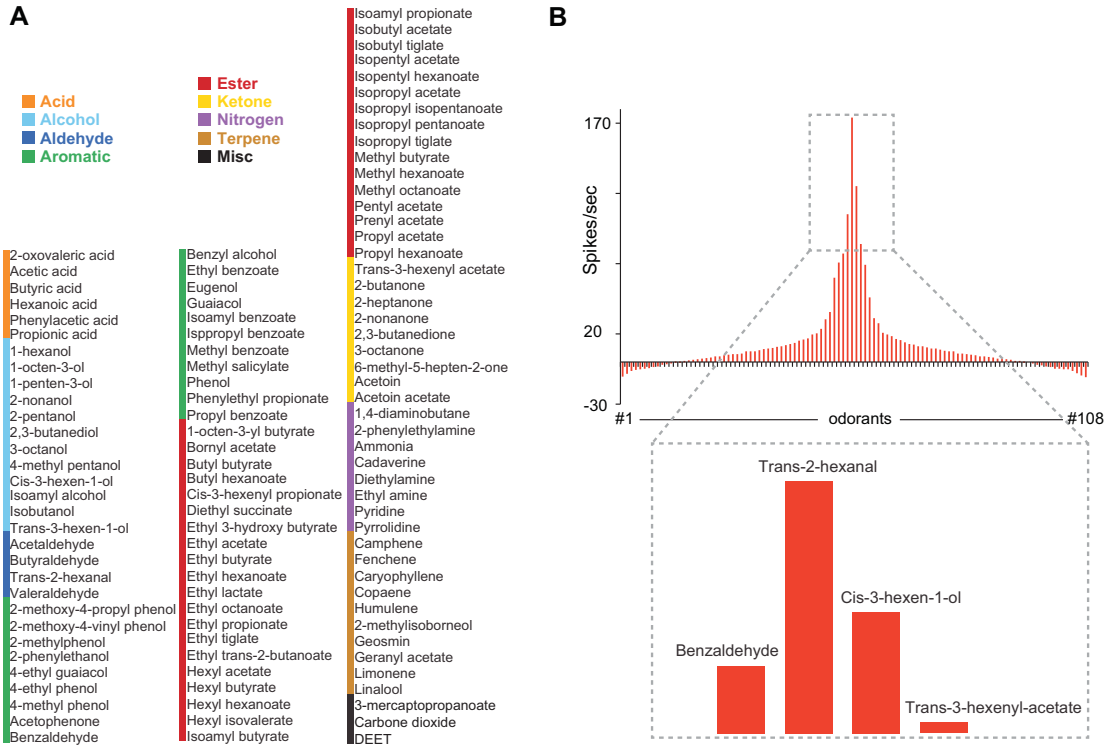

Figure S2. Screened Synthetic Volatiles and Properties of the ab4A Neuron, Related to Figure 3

(A) Screened odorants.

(B) Tuning curve for the ab4A neuron type based on a screen of 103 synthetic substances.

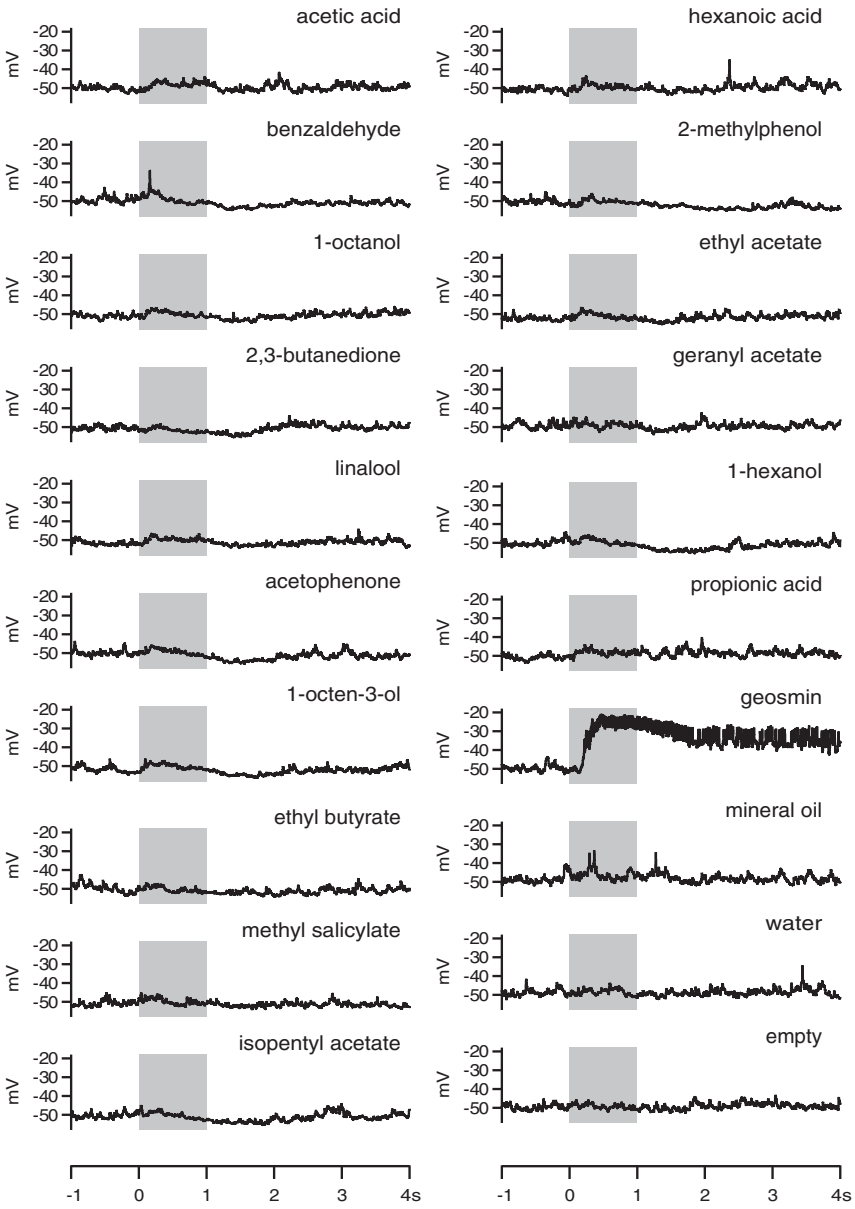

Figure S3. Spike Traces from a DA2 Projection Neuron, Related to Figure 4

Spike traces from a DA2 PN following odor stimulation. Only geosmin elicits any response.

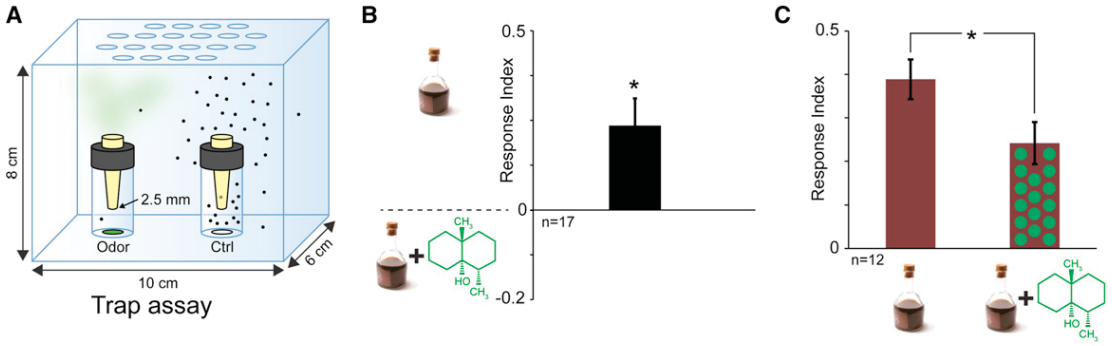

Figure S4. T-Maze and Trap Assay Choice Experiments with a Vinegar and Geosmin Mix, Related to Figure 5

(A) Schematic drawing of the trap assay (Larsson et al., 2004) used in panel (B). For each trial, ~50 flies were placed inside the test boxes. Number of flies in and outside traps was then counted after 24 hr (for further details, see Stökl et al. [2010] and Knaden et al. [2012]).

(B) Response index of wt flies given a choice between balsamic vinegar and balsamic vinegar additionally containing 10^{-3} geosmin in the trap assay. Deviation of the response index against zero was tested with a Student's t test ($p < 0.05$). Error bar represent SEM.

(C) Response indices of wt flies to balsamic vinegar and balsamic vinegar containing geosmin (10^{-3}) in the T-maze assay. Star denotes significant difference (Student's t test $p < 0.05$). Error bars represent SEM.

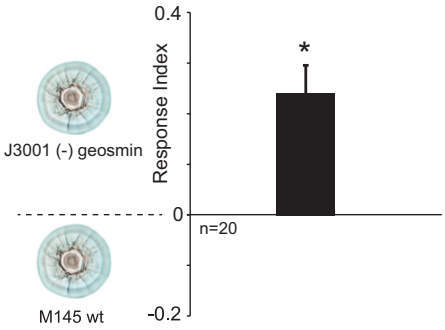

Figure S5. Trap Assay Two-Choice Experiment with WT and Mutant *S. coelicolor*, Related to Figure 6

Response index of flies given a choice between wt (M145) *S. coelicolor* and the J3001 strain in the olfactory choice trap assay (Figure S4A). Star denotes significant difference (Student's t test $p < 0.05$). Error bar represent SEM.

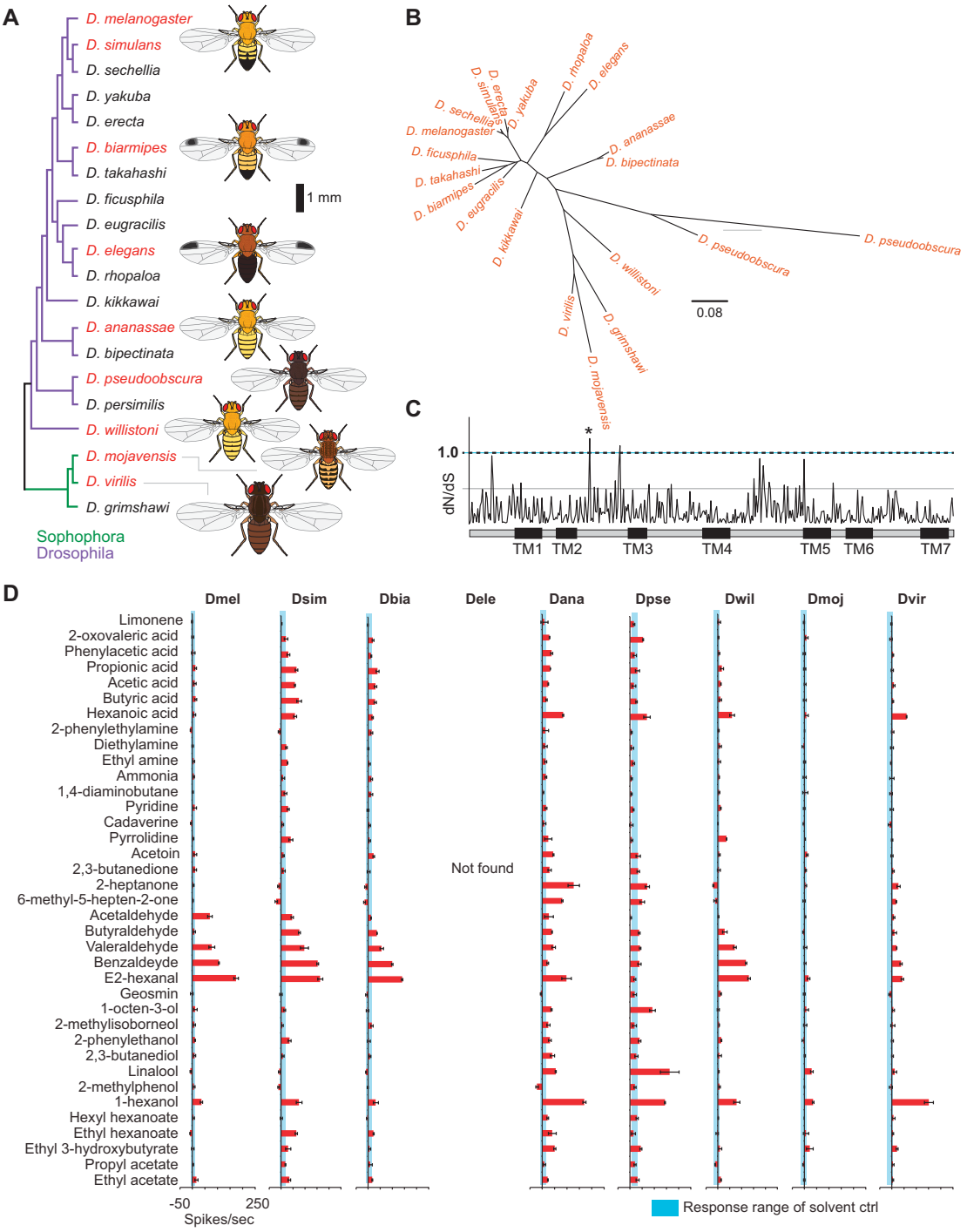

Figure S6. Molecular and Physiological Properties of the ab4 Type Sensillum across Related Drosophilids, Related to Figure 7

(A) Phylogenetic relationship of the examined species.

(B) Phylogenetic tree of *Or56a* orthologs from 19 species. The tree was constructed with RAxML from a Muscle alignment. Scale bar represents number of substitutions per site.

(C) Estimation of the selection pressure acting upon *Or56a*. Plot shows dN/dS ratios (obtained through PAML, model M8) for all codons, here plotted on the sequence of *D. melanogaster*. TM1-7 indicates putative locations of transmembrane domains (estimated with HMMTOP/TMHMM). Star denotes site under significant positive selection (Bayes Empirical Bayes).

(D) Response profile of neurons (n = 3) paired with the geosmin responsive neurons shown in Figure 6. Error bars represent SEM.

MANUSCRIPT II

Olfactory Preference for Egg Laying on Citrus Substrates in *Drosophila*

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Article

Olfactory Preference for Egg Laying on *Citrus* Substrates in *Drosophila*

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Summary

Background: Egg-laying animals, such as insects, ensure the survival of their offspring by depositing their eggs in favorable environments. To identify suitable oviposition sites, insects, such as the vinegar fly *Drosophila melanogaster*, assess a complex range of features. The fly selectively lays eggs in fermenting fruit. However, the precise cues and conditions that trigger oviposition remain unclear, including whether flies are also selective for the fruit substrate itself.
Results: Here, we demonstrate that flies prefer *Citrus* fruits as oviposition substrate. Flies detect terpenes characteristic of these fruits via a single class of olfactory sensory neurons, expressing odorant receptor *Or19a*. These neurons are necessary and sufficient for selective oviposition. In addition, we find that the *Citrus* preference is an ancestral trait, presumably representing an adaptation toward fruits found within the native African habitat. Moreover, we show that endoparasitoid wasps that parasitize fly larvae are strongly repelled by the smell of *Citrus*, as well as by valencene, the primary ligand of Or19a. Finally, larvae kept in substrates enriched with valencene suffer a reduced risk of parasitism.
Conclusions: Our results demonstrate that a single dedicated olfactory pathway determines oviposition fruit substrate choice. Moreover, our work suggests that the fly's fruit preference—reflected in the functional properties of the identified neuron population—stem from a need to escape parasitism from endoparasitoid wasps.

Introduction

For egg-laying animals, such as insects, the capacity to discriminate and choose appropriate sites for oviposition is of profound importance to the fitness of the future generation. The limited mobility of (most) insect larvae also means that the female parent must be able to make an informed decision about any potential oviposition site's future prospects as a suitable home for the larvae. Gravid females accordingly make use of multiple sensory modalities when evaluating the suitability of potential oviposition sites. For example, oviposition site selection in mosquitoes depends upon evaluation of a complex range of chemical and physical factors of their aquatic niches, ranging from, e.g., optical density, pool reflectance, salinity, chemical cues from conspecifics, and the presence of anuran tadpoles to the composition of the surrounding vegetation [1, 2].

The vinegar fly *Drosophila melanogaster*, which utilizes fermenting fruit as breeding substrate, likewise assesses a wide range of factors prior to choosing its oviposition site. Flies are selective, e.g., for (or against) color [3], ethanol and sugar content [4–6], temperature [7], fermentation volatiles [8, 9], endoparasitoid wasps [10, 11], substrate texture [12], and microbial composition [13]. Of the sensory cues involved, olfactory input plays a crucial role. The smell of acetic acid alone acts as a strong oviposition stimulant [14], whereas the smell of geosmin, an indicator of harmful microbes, prevents egg laying [13]. The microbial composition of the potential oviposition substrate is clearly a critical factor; however, whether flies also display partiality with respect to the substrate itself on which the microbes grow, i.e., the fruit, remains unclear. Do flies have an oviposition preference for certain fruits, and are there fruit-produced volatiles that, similar to acetic acid, act as oviposition stimulants?

We here investigated oviposition preference toward fruit in *D. melanogaster*. We find that flies indeed have an innate olfactory preference for certain fruits, preferring *Citrus* spp. and fruits with similar characteristics. We also find that this preference is mediated via a single class of olfactory sensory neurons, dedicated to the detection of terpenes typical of flavedo (i.e., the colored rind found in *Citrus*). The *Citrus* partiality likely reflects an ancestral preference toward specific fruits found in the native African habitat. Finally, we demonstrate that the *Citrus* preference has likely been driven by needs to avoid parasitization from endoparasitoid wasps.

Results and Discussion

Flies Prefer *Citrus* Fruits for Oviposition

We first assessed the egg-laying preference of *Drosophila melanogaster* toward different fruits using a multiple-choice oviposition assay in which flies had unrestricted access to presented fruits (six at a time). Importantly, we screened only ripe, undamaged fruits, to exclude yeast that might influence the flies' choice. In three iterative trials, wild-type (WT) flies consistently chose sweet oranges as oviposition substrate over the 15 other fruits tested (Figure 1A). Flies (n = 30 per trial, 10 trials per treatment, each lasting 24 hr) deposited on average 103.0 ± 51.1 (SD) eggs on the oranges, compared to between 0 and 30.9 ± 20.4 on the other fruits. Flies clearly showed little liking for lemon, not unexpected given the acidity of this fruit. However, the effect of orange could be recapitulated by grapefruit (data not shown), suggesting that except for the most acidic taxa, given a choice, flies will prefer to oviposit on *Citrus* spp. Accordingly, we conclude that flies do not indiscriminately oviposit on any fruit but display a preference for certain fruits, in our screen represented by *Citrus* spp. Since the tested flies had no prior experience with fruit, we further conclude that this preference is innate.

The Oviposition Preference for *Citrus* spp. Is Dependent on Limonene

Flies, like many other insects, rely on their sense of smell to locate objects of importance [15]. Hence, we next sought to

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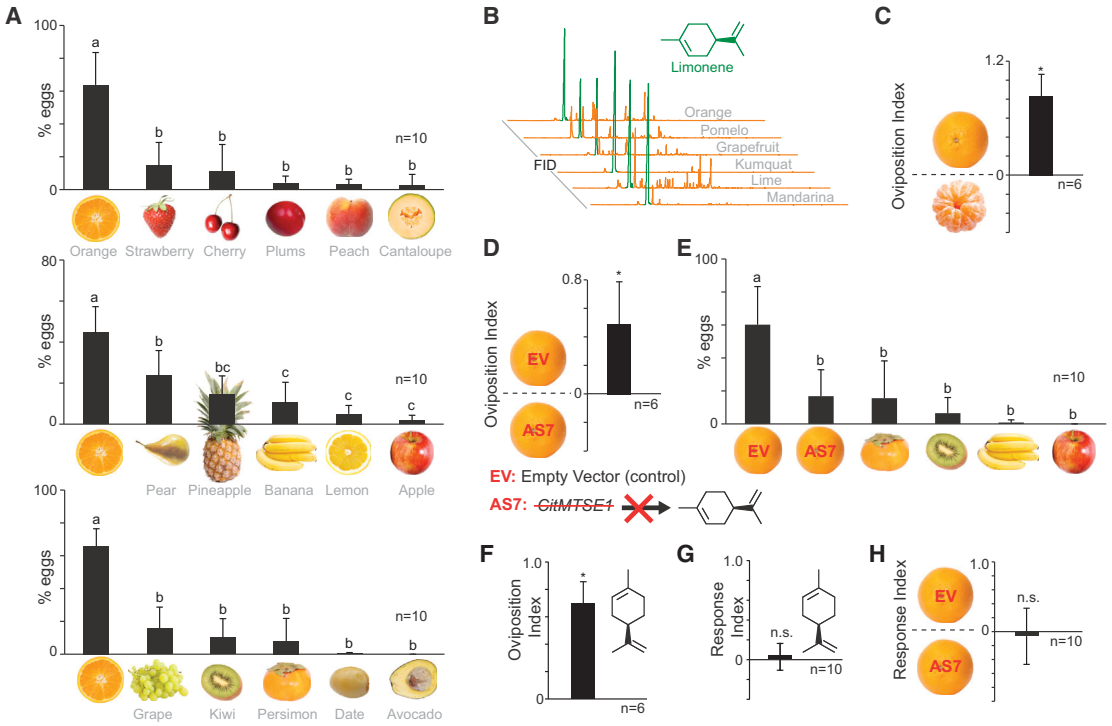

Figure 1. Flies Prefer *Citrus* as Oviposition Substrate

(A) Percentage of eggs deposited on fruits presented in six-way choice oviposition experiments. Error bars represent SEM. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(B) Flame ionization (FID) traces from headspace collections of various *Citrus* varieties. Limonene is the major volatile constituent.

(C) Oviposition index (OI) from a binary choice between intact and peeled oranges. OI = 1 denotes all eggs deposited on intact oranges; OI = −1 denotes all eggs deposited on peeled oranges. Deviation of the OI against zero (no choice) was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(D) OI from a binary choice between oranges transfected with empty vector (EV) and oranges with antisense downregulation of a limonene synthase gene (*CitMTSE1*) (AS7). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(E) Percentage of eggs deposited on fruits in a six-way choice oviposition experiment. Abbreviations are as per (D). Error bars represent SEM. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(F) OI to limonene (10^{-2} dilution). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$).

(G) Response index (RI) to limonene (10^{-2} dilution). Error bars represent SEM. Deviation of the RI against zero was tested by Student's t test ($p < 0.05$).

(H) RI from a binary choice between the orange lines described in (D). Error bars represent SEM. Deviation of the RI against zero was tested by Student's t test ($p < 0.05$).

identify olfactory cues mediating the fruit partiality. In terms of volatile chemistry, *Citrus* fruits are characterized by a high content of terpenes, in particular limonene. This volatile occurs in extraordinary amounts in most *Citrus* varieties [16] (Figure 1B), where it accumulates in the flavedo. The flavedo further contains a plethora of other terpenes in high amounts [16]. In a binary choice oviposition assay [13], flies clearly preferred intact oranges over peeled oranges (Figure 1C), implying that chemicals present in the flavedo are important. To determine the role of limonene, we tested in our binary oviposition assay a transgenic line (AS7) of sweet oranges with reduced limonene content due to antisense downregulation of a key gene involved in limonene synthesis (*CitMTSE1*) [17] against a control line with normal limonene content. Flies strongly preferred the control line (Figure 1D). Likewise, in a multifruit comparison, flies did not choose the AS7 line as egg-laying substrate over other fruits: flies laid as many eggs on the AS7 line as they did on apple, persimmon, kiwi, or banana (Figure 1E). We accordingly conclude that the presence of limonene is necessary for the increased rate of oviposition seen toward *Citrus* fruits.

Is limonene sufficient to induce oviposition? In a binary olfactory choice oviposition assay [13], flies strongly preferred to oviposit on food plates spiked with synthetic limonene (Figure 1F). This result could however also be explained by flies having an innate attraction to limonene, thus spending more time on the baited plate and hence laying more eggs. In other words, limonene could be acting as an oviposition attractant rather than an oviposition stimulant [18]. To exclude this possibility, we examined the behavioral valence of limonene using a modified olfactory trap assay [9, 19]. Limonene was neutral, with flies displaying neither attraction nor repulsion (Figure 1G). Moreover, flies exposed to the odor of the AS7 and empty vector (EV) lines in the olfactory trap assay likewise showed no preference for either genotype (Figure 1H). We hence conclude that volatile limonene by itself is a genuine oviposition stimulant, in a fashion similar to acetic acid [14].

Limonene Is Detected by OSNs Housed in an Antennal Intermediate Sensillum Type

We next sought to identify the olfactory sensory neurons (OSNs) that detect limonene, via a system-wide single-sensillum recording (SSR) screen from all OSN classes found on the third antennal segment and maxillary palps, while stimulating OSNs with limonene. Only antennal intermediate sensillum type 2A (ai2A) neurons [20] responded strongly to

limonene (Figures 2A and 2B). Apart from ai2A, we additionally noted a weaker response to limonene from antennal basiconic sensilla type 9A (ab9A) (Figure 2A). To verify that limonene is detected primarily via the ai2A neurons, we examined the response threshold toward limonene for these two OSNs. Indeed, the limonene detection threshold of ai2A was at least three orders of magnitude lower than that of ab9A (Figure 2C). Thus, we conclude that at ecologically relevant concentrations, the presence of limonene is mediated solely via a pathway receiving input from ai2A OSNs.

We next sought to determine which other compounds the ai2A OSNs might respond to. We tested in our SSR assay 450 synthetic chemicals—a set that contained multiple representatives from all biologically relevant chemical classes (Figure 2D; see also Figure S1 available online). Out of the 450 screened substances, only 5% yielded a response of >50 spikes/s, and only seven compounds produced a firing rate of >100 spikes/s. These seven compounds were all terpenes, as well as sharing other structural features with limonene (Figure 2D). The strongest response was not recorded from limonene but from valencene, another characteristic *Citrus* volatile [21]. To determine the most efficient ligands for ai2A, we subsequently examined dose-response relationships for 28 compounds, a subset that included the most efficient ligands from the initial screen and a range of other terpenes (Figure 2E). The dose-response trials revealed that the most efficient activator for this OSN population was indeed valencene, followed by β-caryophyllene, β-caryophyllene oxide, and limonene oxide, with the latter three showing similar efficiency at activating ai2A (Figure 2E). These three substances, although commonly occurring in nature, are nevertheless typically also found in *Citrus* headspace, in particular limonene oxide [16].

Do the additional ai2A ligands elicit a behavioral response similar to limonene? To address this question, we tested four of the ligands in the oviposition as well as in the olfactory trap assay. Indeed, all of these compounds triggered oviposition (Figure 2F), but no apparent chemotaxis (Figure 2G), and thus similarly act as oviposition stimulants. Moreover, we would also expect that ai2A OSNs are activated by the smell of genuine *Citrus* fruits. Thus, we next used gas chromatography (GC)-linked SSR to stimulate ai2A OSNs with headspace from a range of *Citrus*. As expected, all seven *Citrus* varieties screened strongly activated the ai2A neurons (Figure 2H). We thus conclude that the ai2A OSNs are configured specifically for the detection of terpenes, particularly those associated with *Citrus*.

ai2A Neurons Express Or19a and Target the DC1 Glomerulus

To identify the odorant receptor (OR) underlying the response property of the ai2A neurons, we visualized the activity of antennal lobe (AL) glomeruli using in vivo calcium imaging and delineated the identity of the corresponding OR by virtue of the published map of OR expression in the fly AL [22, 23] (Figure 3A). Stimulation with limonene, valencene, and β-caryophyllene primarily activated a region in the AL corresponding to the DC1 glomerulus (Figures 3B and 3C). In line with the SSR data, we also noted weaker responses to limonene from the D glomerulus (Figure 3C), which is the target of OSNs expressing *Or69a* and housed in the ab9 sensillum [22]. DC1 receives input from OSNs expressing *Or19a* and *Or19b* [22, 23], of which the former has previously been found to bind limonene [24]. Indeed, misexpression of Or19a in Δab3A OSNs [25] endows these neurons with a response profile inseparable from that of ai2A OSNs when stimulated with synthetic volatiles (Figures 3D and 3E), as well as with *Citrus* headspace via GC (Figure 3F). The function of *Or19b*, if any, remains to be elucidated. We accordingly conclude that the terpene responsiveness of the ai2A OSNs is due to *Or19a*.

ai2A OSNs Are Necessary and Sufficient for the Oviposition Preference toward *Citrus*

Are the ai2A neurons necessary for the observed behavior? We next used the temperature-sensitive mutant dynamin *Shibire^{ts}* [26] expressed from the *Or19a* promoter to shut down synaptic transmission in ai2A OSNs. First, we examined the oviposition behavior toward limonene, valencene, and β-caryophyllene. At the restrictive temperature (32°C), flies carrying this construct displayed no oviposition preference toward these compounds (Figure 3G), unlike flies with the same genotype tested at a permissive temperature (25°C) and control lines. Strikingly, thermogenetic silencing of the ai2A neurons also completely abolished the preference for *Citrus* fruit at the restrictive temperature in a binary oviposition choice test with oranges versus plums (Figure 3H). As expected, silencing of the ab9A OSNs, via expression of *Shibire^{ts}* from the *Or69a* promoter, had no effect on the oviposition behavior toward valencene (Figure S1A), or any effect in the oranges-versus-plums oviposition test (Figure S1B).

We next wondered whether activation of this OSN population is sufficient to induce oviposition. We subsequently expressed the temperature-sensitive cation channel *dTRPA1* in the ai2A OSNs, which allowed us to conditionally and specifically activate these neurons at temperatures above 26°C [27]. In a binary choice oviposition assay, flies bearing the *Or19a-Gal4,UAS-dTRPA1* construct preferred to deposit eggs on plates heated to 26°C over plates held at room temperature (20°C), in contrast to parental controls and WT flies, which showed no such preference (Figure 3I). Specific activation of these neurons is hence sufficient to induce oviposition. To further explore the sufficiency of these neurons in guiding oviposition site selection, we again provided flies with the choice to oviposit on either oranges or plums, but now adding valencene—the key ligand for *Or19a*—to the plums. Indeed, adding this volatile alone to the plums abolished the *Citrus* preference (Figure 3J). In summary, we conclude that *Or19a* is both necessary and sufficient for the oviposition preference toward *Citrus*.

Citrus Fruits Are Not the Ancestral Host of *D. melanogaster*

Citrus fruits are native to Southeast Asia [28], whereas *D. melanogaster* stems from Africa [29]. How can *D. melanogaster* have evolved a tight association with fruits that it has not coevolved with? One explanation could be that the preference for *Citrus*, and in turn the tuning of *Or19a* toward volatiles of a *Citrus* character, represents an ancestral trait. The *melanogaster* species subgroup comprises an African offshoot of a Southeast Asian radiation. One could envision that the ancestral Asian population from which *D. melanogaster* stems utilized *Citrus*, and that this preference, reflected in the olfactory makeup, was retained when Africa was colonized during late Miocene [30]. Once in Africa, the colonists would have found fruits with chemical (and physical) properties similar to those of *Citrus*. A GC-SSR comparison of 13 species from across the subgenus *Sophophora* (Figures S3A and S3B), with orange headspace as stimulus, demonstrated that there are indeed Asian relatives with ai2A

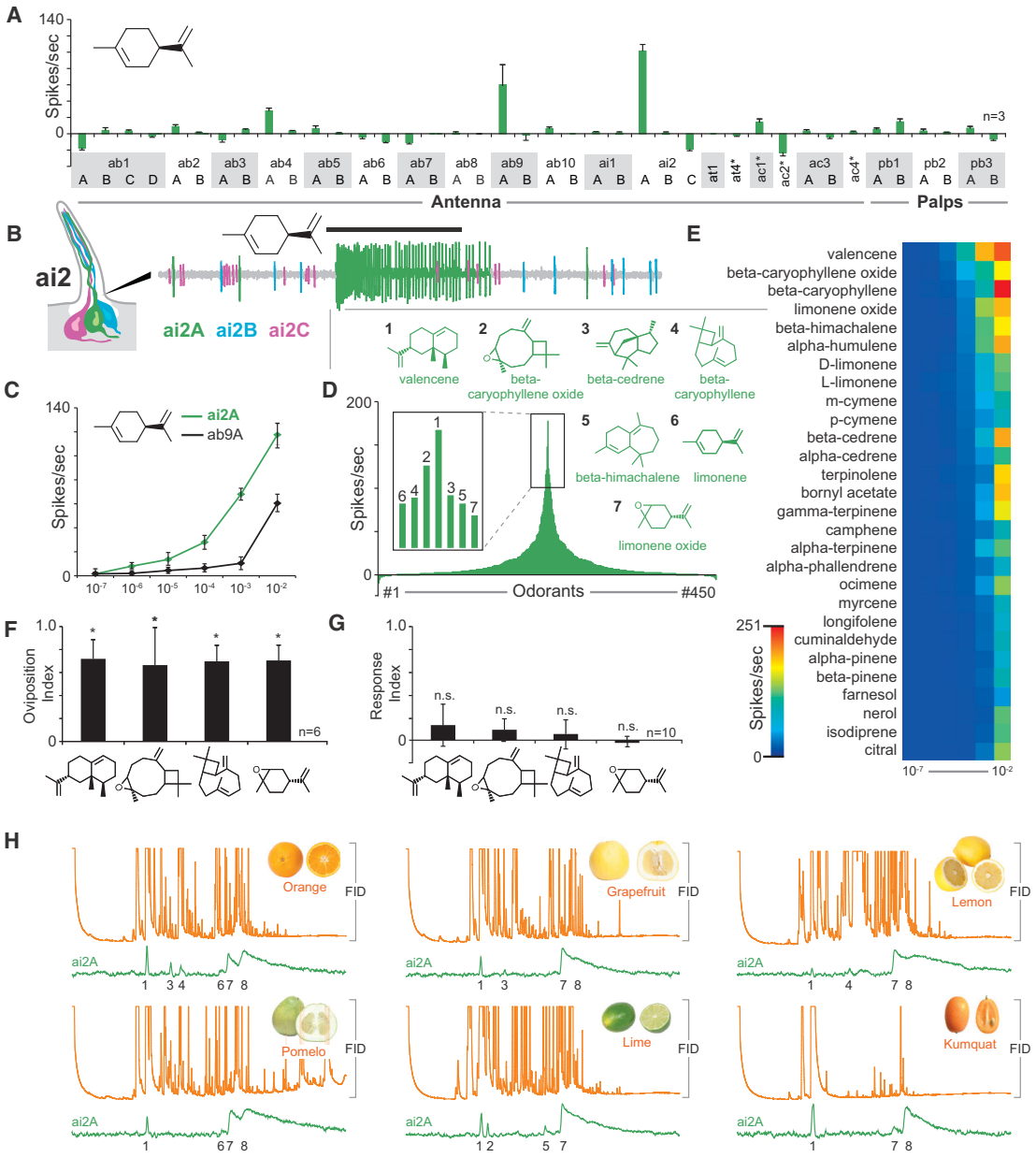

Figure 2. *Citrus* Odorants Are Detected by the ai2A Neurons

(A) Single-sensillum recording (SSR) measurements from all olfactory sensilla, with limonene (10^{-3} dilution) as a stimulus. ab, antennal basiconic sensilla (s.); ac, antennal coeloconic s.; at, antennal trichoid s.; ai, antennal intermediate s.; pb, palp basiconic s. Asterisks denote that activity from individual OSNs was not separated. Error bars represent SEM.

(B) Representative SSR traces from an ai2 sensillum. The larger-amplitude spiking neuron, i.e. ai2A, responds to limonene (10^{-3} dilution). The duration of stimulus delivery (0.5 s) is marked by the black bar.

(C) Dose-response curve from ai2A neurons toward limonene. Error bars represent SEM.

(D) Tuning curve for the ai2A neuron type based on a screen of 450 synthetic substances (10^{-2} dilution). Error bars represent SEM.

(E) Heatmap based on dose-response profiles of ai2A neurons toward 28 compounds.

(F) Oviposition indices (OI) to valencene, β-caryophyllene, β-caryophyllene oxide, and limonene oxide. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(G) Response indices (RI) from olfactory trap assay experiments toward the same compounds as in (F). Deviation of the RI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(H) Representative gas chromatography (GC)-linked SSR measurements from ai2A neurons. The orange trace represents the FID, photos depict the screened odor sources, and the green trace depicts the simultaneously recorded neural activity of ai2A neurons. Numbers refer to the identity of active FID peaks (as determined via GC-MS): 1, limonene; 2, γ-terpinene; 3, limonene oxide; 4, unidentified; 5, γ-elemene; 6, β-cubebene; 7, β-caryophyllene; 8, valencene.

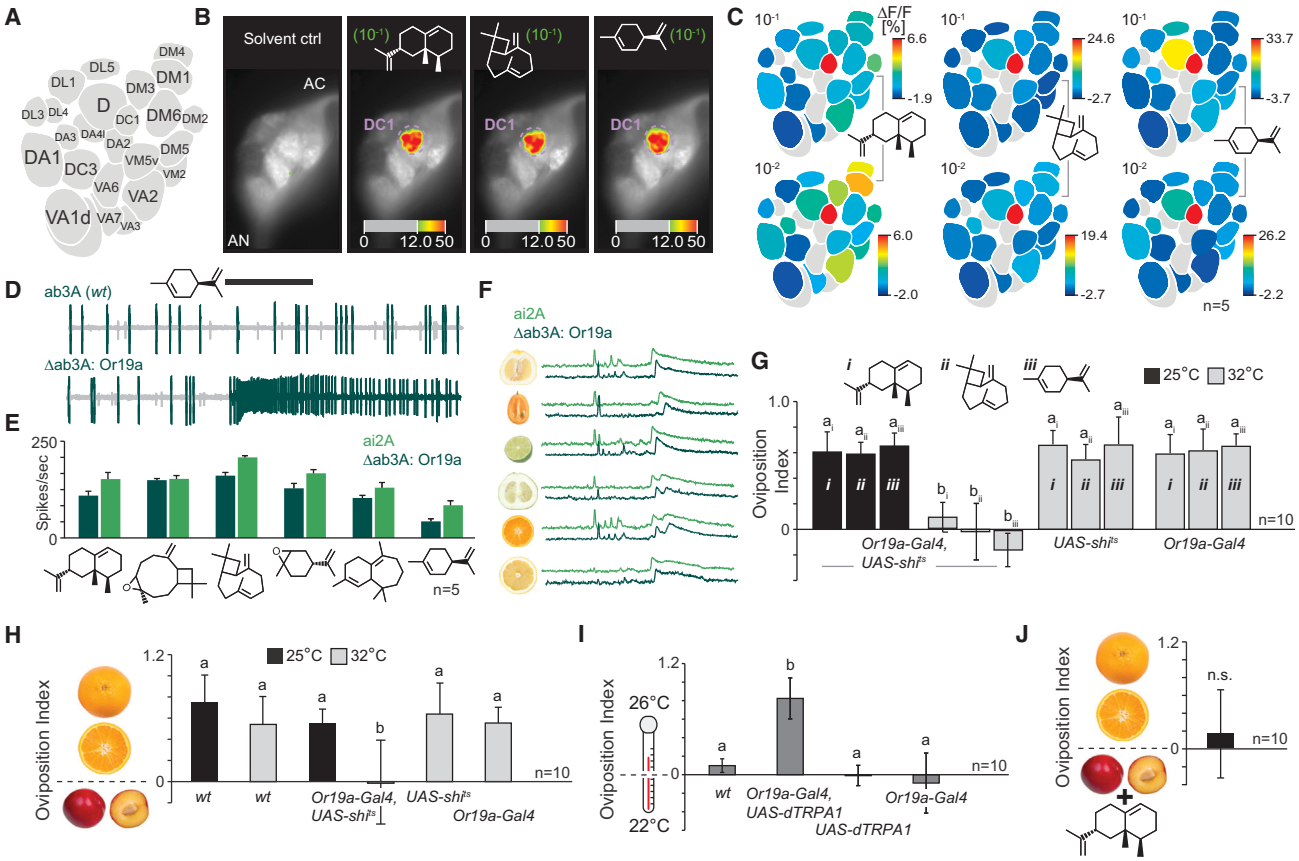

Figure 3. *Or19a* Is Necessary and Sufficient for the *Citrus* Preference

(A) Glomerular atlas of the antennal lobe (AL).

(B) False color-coded images showing solvent-induced and odorant-induced calcium-dependent fluorescence changes in the AL of a fly expressing the activity reporter GCaMP3.0 from the *Orco* promoter. AC, antennal commissure, AN, antennal nerve.

(C) Odor-induced activity plotted on schematic ALs (average % DF/F).

(D) Representative SSR traces from measurements of WT ab3 (above) and *Δab3:Or19a* (*Δhalo;Or22a-GAL4/UAS-Or19a*) (below) stimulated with limonene (10^{-3}). The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(E) Quantified SSR responses toward valencene, β-caryophyllene, β-caryophyllene oxide, limonene oxide, β-himachalene, and limonene from ai2A (green) and Δab3:Or19a OSNs (dark green). Error bars represent SEM.

(F) Representative GC-SSR traces from ai2A and *Δab3:Or19a* OSNs stimulated with a variety of *Citrus* spp. Color coding is as per (E).

(G) OIs to valencene, β-caryophyllene, and limonene (all at 10^{-1}) of flies expressing *Shibire^{ts}* from the *Or19a* promoter and corresponding parental lines. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(H) OIs of flies expressing *Shibire^{ts}* from the *Or19a* promoter and corresponding parental lines presented with a choice to oviposit on either oranges or plums. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(I) OIs of flies expressing *dTRPA1* from the *Or19a* promoter, the corresponding parental lines, and WT flies in an oviposition assay with a choice between 22°C and 26°C. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(J) OIs of flies confronted with a choice between oranges and plums spiked with valencene (10^{-3}). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

OSNs tuned as in *D. melanogaster* (Figure S3C). The species most similar to *D. melanogaster* is in fact *D. bipectinata*, a widespread species occurring from India to Samoa [31]. Although the ecology of this species is poorly known, given an oviposition choice between oranges and plums, *D. bipectinata* also strongly preferred oranges (oviposition index 0.97 ± 0.05 [average ± SD]; $p = 0.0001$, Student's t test against zero [1.0 = full preference for oranges]). It is hence not inconceivable that the *Citrus* partiality, and tuning of the ai2A OSNs, constitutes an ancestral trait that has remained conserved in the lineage leading to *D. melanogaster*.

Irrespective whether the observed behavior is an ancestral attribute or was acquired independently after the colonization of Africa, there should presumably be fruits with chemical properties similar to those of *Citrus* within the native range of *D. melanogaster*. We subsequently went to the field and obtained headspace collections from a variety of native African noncultivated fruits (n = 6) and examined the GC-SSR activity pattern of ai2A OSNs. We then compared the responses triggered by these fruits to those elicited by a host of other non-*Citrus* (n = 12) and the previously examined *Citrus* (n = 7). With two exceptions, none of the non-*Citrus* varieties elicited any noticeable responses from the ai2A neurons (Figure 4A). Stimulation with giant yellow mulberry (*Myrianthus arboreus*) triggered a single response (unidentified peak), whereas stimulation with headspace from African squirrel nutmeg (*Monodora tenuifolia*) yielded a response pattern akin to that of *Citrus* (Figure 4B). Flies given a binary oviposition choice

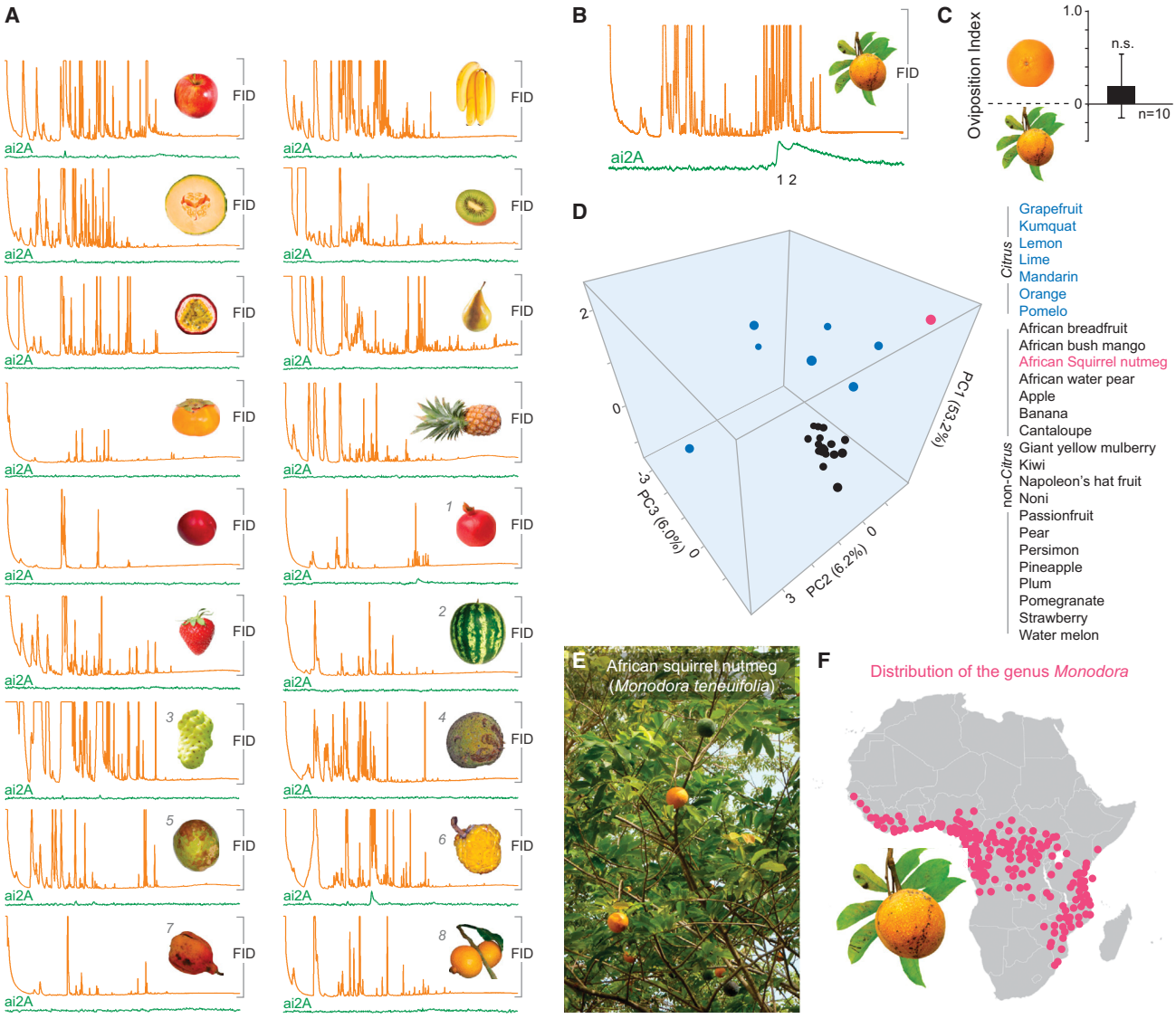

Figure 4. The *Citrus* Preference of *D. melanogaster* Is an Ancestral Trait

(A) Representative GC-SSR traces from *D. melanogaster* stimulated with a range of fruit. Gray numbers indicate (1) pomegranate, (2) watermelon, (3) noni *Morinda citrifolia*, (4) African breadfruit *Treculia africana*, (5) African bush mango *Irvingia wombulu*, (6) African giant mulberry *Myrianthus arboreus*, (7) Akee apple *Blighia sapida*, (8) Napoleon's hat fruit *Napoleona imperialis*.

(B) GC-SSR trace from *D. melanogaster* stimulated with headspace of African squirrel nutmeg. Numbers refer to identity of active FID peaks, as determined via GC-MS. 1, β-caryophyllene; 2, unidentified terpene.

(C) Oviposition index from a binary choice between orange and African squirrel nutmeg. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(D) Three-dimensional principal component analysis plot based on the GC-SSR traces in (A) and (B).

(E) The African squirrel nutmeg in nature (photo by D.B.).

(F) Distribution of the genus *Monodora*. Image adapted from African Plant Database (www.ville-ge.ch/musinfo/bd/cjb/africa/).

test between *Monodora* and oranges showed no significant preference either way (Figure 4C). The similarity could also be seen in a three-dimensional principle component analysis plot based on the response pattern (Figure 4D), where all non-*Citrus*, with the exception of African squirrel nutmeg, cluster together separately from *Citrus*. African squirrel nutmeg also shows an overall likeness to oranges (Figure 4E) that extends to color, shape, and size. Similar to *Citrus*, *Monodora* fruits have a thick epicarp, where presumably the terpenes triggering activity from ai2A neurons accumulate. Are *Monodora* fruits then the ancestral breeding substrate of *D. melanogaster*? Probably not. First of all, members of the genus *Monodora* are restricted to the tropical rainforest zone (Figure 4F). The presumed evolutionary cradle of *D. melanogaster*, however, lies in drier habitats further south, possibly in the Miombo forest zone [32]. Moreover, although the flies readily laid eggs on these fruits, the mesocarp of *Monodora* fruits is quite dry in comparison with fruits typically utilized by *D. melanogaster*, making the suitability of these fruits as larval substrate questionable. Nevertheless, the African squirrel nutmeg serves as proof of principle that there are fruits in Africa with properties similar to those of *Citrus*.

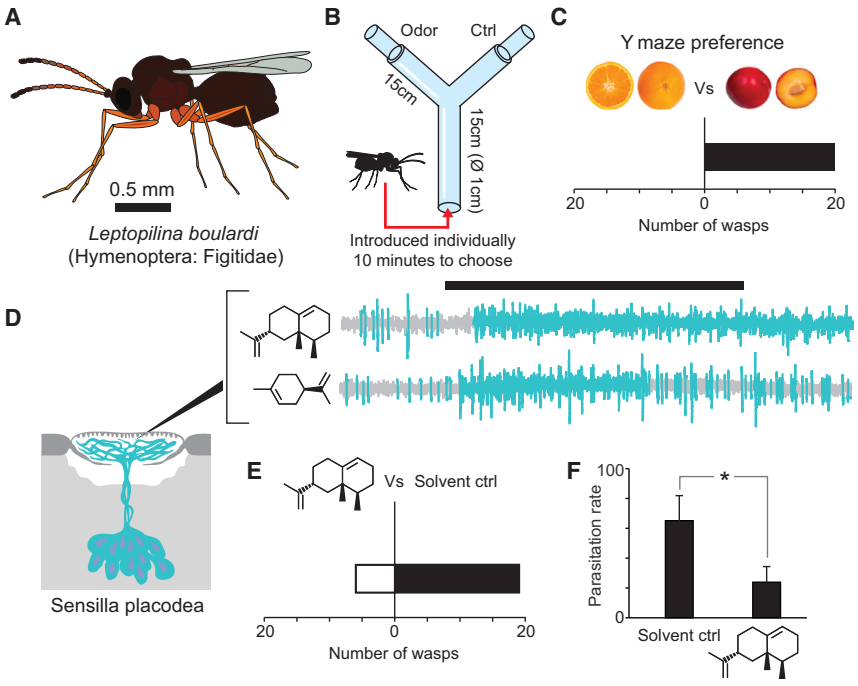

Figure 5. *Citrus* Volatiles Confer Protection against Endoparasitoid Wasps

(A) Schematic drawing of the endoparasitoid wasp *Leptopilina boulardi*, a major larval parasite of *D. melanogaster*.

(B) Schematic drawing of the Y maze olfactory assay used for the wasp behavioral experiments.

(C) Number of wasps choosing oranges versus plums, both infected with fly larvae, in Y maze choice experiments (n = 20).

(D) Representative SSR traces from antennal sensilla placodea of *L. boulardi*, stimulated with valencene and limonene, respectively (at 10^{-2} dilution). As in other Hymenoptera, individual OSNs cannot be discerned. The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(E) Number of wasps moving toward valencene or solvent control in Y maze choice experiments (n = 25). Deviation against even distribution was tested by χ^2 test ($\chi^2 = 6.8$, $p < 0.01$).

(F) Parasitization rate, measured as the number of emerging flies divided by number of eggs laid on plates inoculated with either valencene or solvent control. Asterisk denotes significant difference by Student's t test ($p < 0.05$). Error bars represent SEM.

Identifying the actual ancestral breeding substrate will be a daunting task involving also finding genuinely wild populations of *D. melanogaster*, a feat no one has accomplished so far [29]. The present work, however, provides clear hints as to the characteristics of the ancestral fruit substrate, which should narrow down the search.

Citrus Confers Protection against Endoparasitoid Wasps

Why do *D. melanogaster* then prefer fruits with *Citrus*-like characteristics as oviposition substrate? One reason could be that fruits with a thick epicarp offer protection from parasitoids. In the wild, parasitization from endoparasitoid wasps is a major cause of mortality in drosophilid flies, and in *D. melanogaster*, populations with a >80% parasitization rate have been reported [33]. *Citrus*-like fruits may be advantageous for the reason that the thick rind would form a physical barrier against probing wasps. If a hard epicarp constitutes an obstacle in the parasitization process, we could assume that wasps avoid searching out larvae in fruits with these characteristics. To investigate this, we next examined olfactory-guided behavior of *Leptopilina boulardi* (Figure 5A), an endoparasitoid wasp specialized upon *D. melanogaster* [34], in a Y maze assay (Figure 5B). Confronted with a choice of oranges or plums in the Y maze, wasps made the opposite choice as compared to flies, strongly preferring the smell of plums (Figure 5C). The innate preference of the wasps is accordingly contradictory to that of flies. We next wondered whether the evident repulsion caused by oranges is mediated via the same flavedo terpenes that trigger oviposition in flies. We first used SSR to examine whether wasps can smell these compounds. Recordings from sensilla placodea of the wasps, which contain multiple OSNs (>20) [35], revealed increased spike firing from an unknown number of OSNs in response to stimulation with valencene and limonene (Figure 5D). Having confirmed that wasps are equipped with the machinery to detect these compounds, we next examined the behavioral effect in the Y maze assay. The wasps clearly avoided valencene (Figure 5E). We thus conclude that wasps are repelled by the odor of *Citrus* and that the repellency resides in part or wholly with the presence of terpenes. A fly depositing eggs in a substrate containing valencene and similar terpenes should hence run a reduced risk of having its offspring parasitized. To test this notion, we placed second-instar fly larvae (n = 100 for each treatment) on plates with either fly food baited with valencene or solvent control (mineral oil) added. We thereupon exposed the larvae to ten female wasps for 48 hr, after which we transferred the larvae to vials and then waited for either adult parasitoids or flies to emerge. Indeed, larvae maintained on valencene suffered a significantly decreased rate of parasitism as compared to those maintained on plates with solvent only (Figure 5F). In summary, the *Citrus* preference of flies is presumably a consequence of the lowered parasitization risk conferred by this type of breeding substrate.

Conclusion

We demonstrate that flies prefer fruits with *Citrus* characteristics as oviposition substrate. We show that this preference is mediated via a single class of OSNs expressing *Or19a*, which is both necessary and sufficient for this behavior. In addition, we find that the *Citrus* preference is an ancestral trait, presumably representing an adaptation to fruits found within the native African habitat. Moreover, we show that endoparasitoid wasps—parasites upon fly larvae—are strongly repelled by the smell of *Citrus*, as well as by valencene, the primary ligand of *Or19a*. Finally, larvae maintained on substrates enriched with valencene suffer a reduced risk of parasitism.

Choosing where to lay eggs is a complex behavior that relies upon input from multiple sensory modalities. Although the choice requires complex sensory input overall, our findings suggest that a limited number of olfactory pathways are involved in oviposition site selection. As we show, oviposition preference toward the fruit substrate itself is in fact mediated via only a single olfactory channel. Even though flies choose

to preferentially oviposit on *Citrus*, flies are evidently able to utilize a wide variety of fruits [15, 36]. In nature, flies oviposit in fermenting fruit, where other signals additionally come into play, guiding oviposition site selection. In terms of olfactory cues, the presence of acetic acid is clearly an important factor [14] that presumably serves as a fermentation indicator to the flies. The pathway being fed by input to the ai2A neurons accordingly acts in concert with other circuits—olfactory as well as taste, visual, and tactile—in guiding oviposition site choice. Future work will need to decipher the relative roles of each of these stimuli in mediating this complex behavior.

Experimental Procedures

Fly Stocks

All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the UCSD Drosophila Stock Center (https://stockcenter.ucsd.edu/info/welcome.php). Transgenic lines were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/), except for *Δhalo;Or22a-GAL4/UAS-Or19a*, which was a gift from J.R. Carlson (Yale University). The *Leptopilina boulardi* strain (established from individuals wild caught in southern France) was a kind gift from J. Stökl (Universität Regensburg).

Stimuli and Chemical Analysis

All synthetic odorants tested were acquired from commercial sources (Sigma, www.sigmaaldrich.com, and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery followed Stökl et al. [9]. The headspace collection of volatiles was carried out according to standard procedures. The transgenic orange lines were gifts from L. Peña (Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias). GC stimulation analysis was performed as described previously [9, 13].

Behavioral Assays

Trap assay experiments were performed as described previously [9], with response index (RI) calculated as (O − C)/T, where O is the number of flies in the baited vial, C is the number of flies in the control vial, and T is the total number of flies used in the trial. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). Oviposition experiments were carried out as described in Stensmyr et al. [13]. Oviposition index was calculated as (O − C)/(O + C), where O is the number of eggs on a baited plate and C is the number of eggs on a control plate. Y maze experiments with wasps were performed as outlined in Figure 5B. For the *dTRPA1* experiments, oviposition plates were placed on silicon heat mats (RS Components, http://www.rs-components.com/index.html) connected to PT100 temperature sensors and a Siemens LOGO! control module (www.siemens.com).

Physiology and Morphology

SSR measurements were performed as described previously [13]. Functional imaging of odor-induced glomerular activity was conducted as outlined in Stökl et al. [9].

Supplemental Information

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.047.

Acknowledgments

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Current Biology, Volume *23*

Supplemental Information

Olfactory Preference for Egg Laying on *Citrus* Substrates in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Sophie Kromann, Deni Bown, Ylva Hillbur, Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

Supplemental Inventory

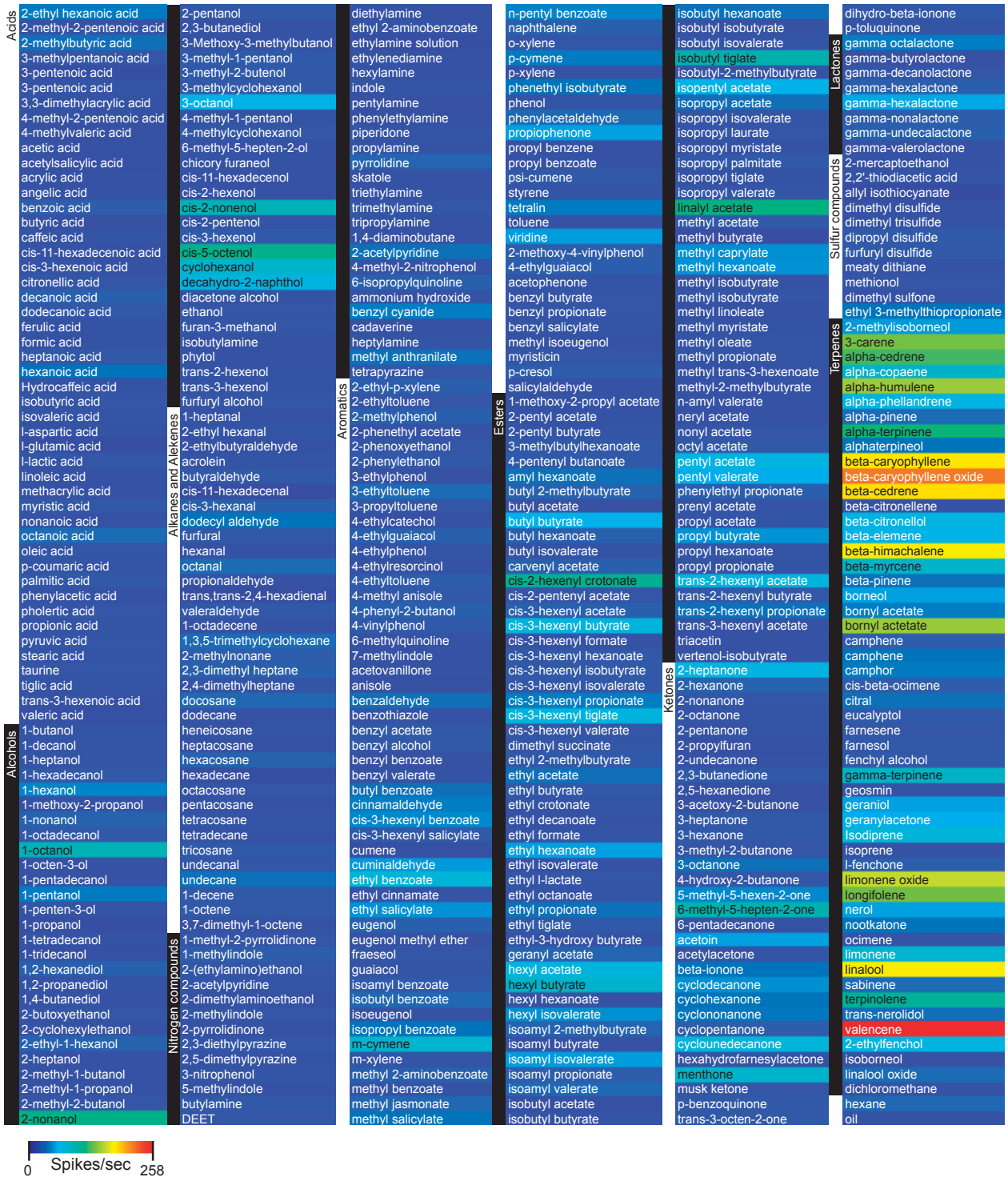

Figure S1 (related to Figure 2).
Heatmap based on responses of ai2A neurons towards 450 screened compounds.

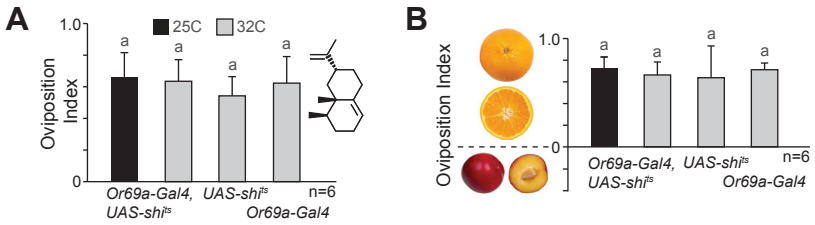

Figure S2 (related to Figure 3).
(A) Oviposition index (OI) to valencene (10^{-2}) of flies expressing *Shibire^{ts}* from the *Or69a* promoter and corresponding parental lines. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.
(B) OIs of flies expressing *Shibire^{ts}* from the *Or69a* promoter and corresponding parental lines presented with a choice to oviposit on either oranges or plums. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

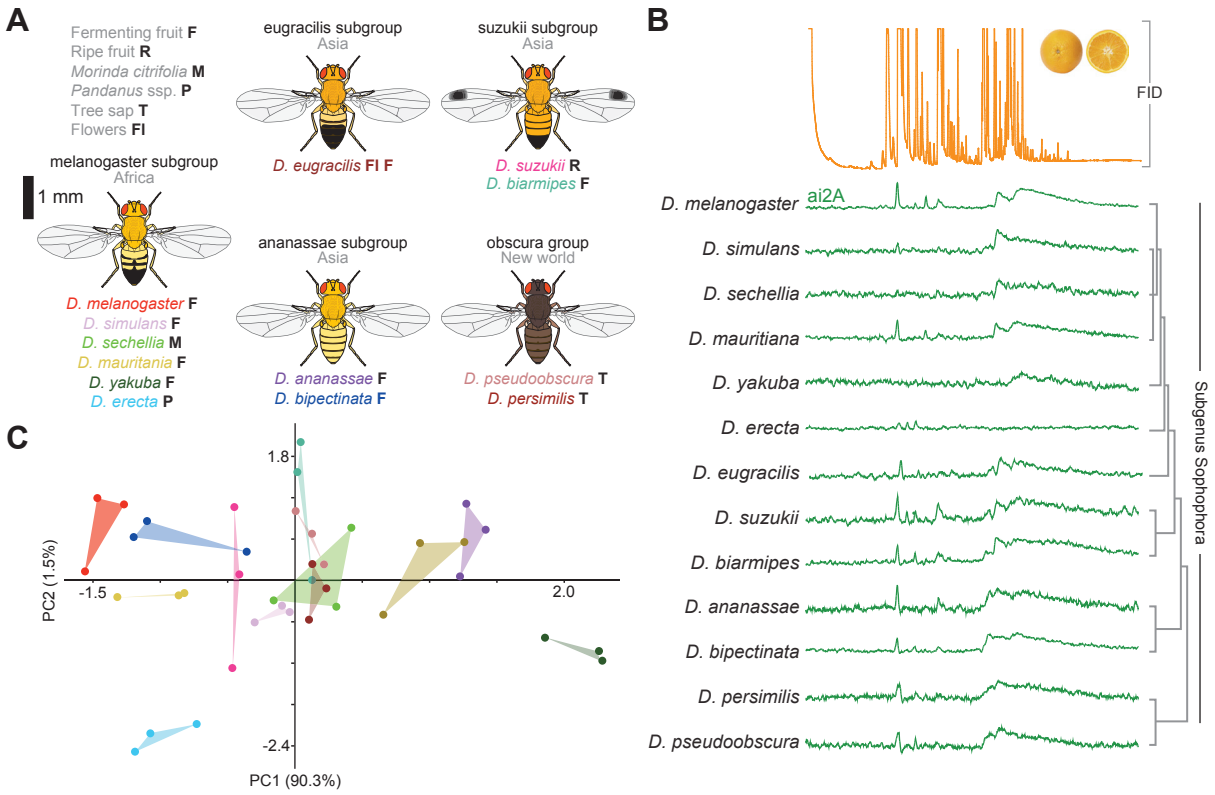

Figure S3 (related to Figure 4).
(A) Examined drosophilid species (subgenus *Sophophora*), sorted according to taxonomic relationship and with breeding substrate indicated.
(B) Representative GC-SSR measurements from 13 species of flies, stimulated with the same orange headspace sample. Phylogenetic relationships of the examined species are given on the right-hand side.
(C) Two-dimensional principal component analysis plot based upon GC-SSR response profiles of 13 species of drosophilids towards orange headspace; exemplified in (B). Color code as in panel (A).

MANUSCRIPT III

Host Plant-Driven Sensory Specialization in *Drosophila erecta*

Jeanine Linz, Amelie Baschwitz, Antonia Strutz, Hany K. M. Dweck, Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

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Host plant-driven sensory specialization in *Drosophila erecta*

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Finding appropriate feeding and breeding sites is crucial for all insects. To fulfil this vital task, many insects rely on their sense of smell. Alterations in the habitat—or in lifestyle—should accordingly also be reflected in the olfactory system. Solid functional evidence for direct adaptations in the olfactory system is however scarce. We have, therefore, examined the sense of smell of *Drosophila erecta*, a close relative of *Drosophila melanogaster* and specialist on screw pine fruits (*Pandanus* spp.). In comparison with three sympatric sibling species, *D. erecta* shows specific alterations in its olfactory system towards detection and processing of a characteristic *Pandanus* volatile (3-methyl-2-butenyl acetate, 3M2BA). We show that *D. erecta* is more sensitive towards this substance, and that the increased sensitivity derives from a numerical increase of one olfactory sensory neuron (OSN) class. We also show that axons from these OSNs form a complex of enlarged glomeruli in the antennal lobe, the first olfactory brain centre, of *D. erecta*. Finally, we show that 3M2BA induces oviposition in *D. erecta*, but not in *D. melanogaster*. The presumed adaptations observed here follow to a remarkable degree those found in *Drosophila sechellia*, a specialist upon noni fruit, and suggest a general principle for how specialization affects the sense of smell.

1. Introduction

Because the sense of smell directly interfaces with the environment, it is an ideal system to study adaptive responses to altered environmental conditions and shifts in habitat preference. An animal exposed to a novel environment or niche will presumably alter its olfactory system over evolutionary time to encompass the composition of new chemical volatiles. Insects are well suited for this line of study, because they possess a rich repertoire of odour-guided behaviours (such as mating and breeding), and their nervous system is accessible as well as numerically reduced compared with the nervous system of vertebrates [1].

The Seychelles endemic *Drosophila sechellia* (*melanogaster* species subgroup, subgenus *Sophophora*)—a close relative of the laboratory work-horse *D. melanogaster*—is a well-known model system for studying questions relating to adaptive host specialization, particularly regarding the olfactory system [2–8]. The *D. sechellia*–*Morinda* system has, however, a number of shortcomings as a model of host plant-driven sensory specialization. Ample evidence suggests that *D. sechellia* has a very small effective population size [9–11], accordingly, observed changes to its chemosensory makeup may be the results of random processes (such as genetic drift) rather than adaptations. In addition, there are also valid concerns over the antiquity of the *Morinda* association ([12]; but see [13]). In order to shed further light on the mechanisms underlying the evolution and adaptation of olfactory systems and to pinpoint processes involved in specialization, we have here investigated the *Drosophila erecta*–*Pandanus* association, the second specialized insect–host system of the *melanogaster* species subgroup.

Drosophila erecta is endemic to gallery forests of west-central Africa (Ivory Coast, Nigeria, Cameroon and Congo) and specializes on ripe fruits of

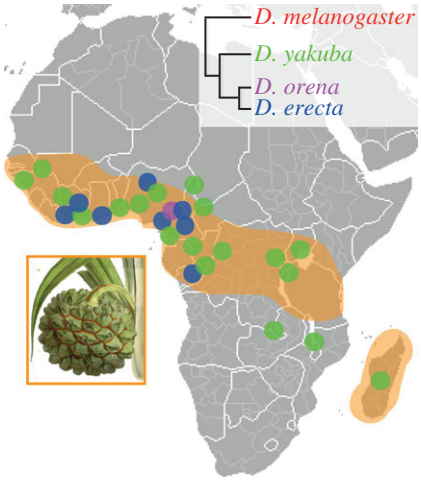

Figure 1. Geographical distribution of *D. erecta*, its sympatric siblings and the genus *Pandanus* in tropical Africa. The four *melanogaster* sibling species differ in their ecology, distribution and phylogenetic relationship. *Drosophila erecta* occurs in swampy and coastal habitats of western-central Africa, and uses fresh *Pandanus* spp. fruits as a food source and breeding site. Its closest relative, *Drosophila orena* (known from a single collection event on Mount Lefo, Cameroon), has an unknown ecology. *Drosophila melanogaster* (cosmopolitan) and *D. yakuba* (endemic to tropical Africa) are generalists. Map by courtesy of Wikipedia; modified from ([15,16]; http://www.mobot.org). *Pandanus candelabrum* image adapted from an original illustration. Reproduced with the kind permission of the Director and the Board of Trustees, Royal Botanic Gardens, Kew.

Pandanus spp. Parkinson (Pandanaceae; [14,15]; figure 1). The oft-mentioned specialization of *D. erecta* towards a single host—*Pandanus candelabrum*—is not correct. The confusion stems from the fact that many *Pandanus* species are taxonomically very hard to distinguish [17]. From the 20 to 24 described *Pandanus* species that occur in continental tropical Africa [15,17], *D. erecta* uses at least three, and is presumably able to use fruits from all *Pandanus* species that bear fleshy syncarps [18–20]. The geographical distribution of *D. erecta* also largely overlaps with the occurrence of the genus *Pandanus* in Africa (figure 1). *Pandanus* trees fruit only once a year over a period of about two months, an attribute that has resulted in *D. erecta* being termed a seasonal specialist [15]. When *Pandanus* fruits are available, *D. erecta* can be viewed as a specialist; however, in times of *Pandanus* shortage, *D. erecta* switches to other food sources and breeding sites. During a 40-month study, Lachaise & Tsacas [14] found a small number of *D. erecta* on fungi, *Ficus capensis* fruits and, once, on a palm bud. In another study [15], a handful of *D. erecta* (but many *D. melanogaster* and *Drosophila yakuba*) were trapped in banana baits between two *Pandanus* fruiting events.

In contrast to *D. erecta*, its sibling *D. sechellia* relies exclusively on fruits of the noni tree (*Morinda citrifolia*, Rubiaceae) for feeding and breeding [19,21]. Fresh noni fruits are hostile to most drosophilids, including its generalist cosmopolitan relatives *D. melanogaster* and *Drosophila simulans*. *Pandanus* syncarps are also visited by other drosophilids, including the sympatric sibling species *D. yakuba* (figure 1), a generalist that prefers *F. capensis* (Moraceae), *Theobroma cacao* (Malvaceae) and *Landolphia hirsuta* (Apocynaceae) [22]. Although, *D. yakuba* (like most of the other visiting drosophilids) uses *Pandanus* fruits only for feeding and not for breeding [14]. By contrast, nothing is known about the ecology of its closest relative, *Drosophila orena*, which is only known from a single collection event on Mount Lefo, Cameroon.

Here, we ask whether the specialized lifestyle of *D. erecta* is reflected in its olfactory system. We demonstrate that the *D. erecta*–*Pandanus* association has resulted in alterations in the olfactory system, similar to those found in *D. sechellia*. Specifically, we show that *D. erecta*, in contrast to its non-specialized sympatric relatives *D. orena*, *D. yakuba* and *D. melanogaster*, is more sensitive towards the *Pandanus* volatile 3-methyl-2-butenyl acetate (3M2BA; also known as prenyl acetate). The enhanced sensitivity is accomplished by increasing a specific class of olfactory sensory neurons (OSNs); intriguingly, this is the same class of OSNs affected in *D. sechellia*. Moreover, we show that the numerical increase in the periphery is accompanied by the formation of enlarged glomeruli in the antennal lobes (ALs), the first olfactory neuropil in the fly brain. Furthermore, 3M2BA triggers oviposition in *D. erecta*.

2. Material and methods

(a) Flies

Wild-type flies were obtained from the *Drosophila* Species Stock Centre (https://stockcenter.ucsd.edu): *D. erecta* (14021–0224.01), *D. orena* (14021–0245.01) and *D. yakuba* (14021–0261.01); except for *D. melanogaster* (wild-type Berlin). For functional imaging, transgenic fly lines of *D. melanogaster* used were Orco-Gal4 [23] and UAS-G-CaMP3.0 [24]. *GCaMP3.0* flies were kept for 36 h at 29°C before experiments to enhance UAS-reporter gene expression. All flies were reared on standard cornmeal medium at 25°C, except for *D. orena* (18°C), 12 L : 12 D photoperiod and 70 per cent relative humidity. All experiments were carried out with mated female flies, 4–10 days post-eclosion, except for oviposition experiments.

(b) Fruit headspace collections

Ripe *Pandanus* sp. fruits were wrapped in polyester bags (Toppits Bratschlauch, Germany), and volatiles were trapped with Super Q adsorbent filters (30 mg; Alltech, Deerfield, IL, USA). Sample collection was done for 6 h (1.0 l min^{-1}) using a vacuum pump (Apex Pro, Casella, UK). Adsorbed volatiles were desorbed by eluting filters with 300 µl dichloromethane (DCM, 99%; Roth). Samples were stored at −20°C.

(c) Chemical analysis

Pandanus fruit volatiles were analysed by gas chromatography-mass spectrometry (GC-MS) (Agilent 6890 GC, and 5975 MS). The GC was equipped with a non-polar HP5 column (30 m × 0.25 mm ID, 0.32 µm film thickness; Agilent) with helium as a carrier gas (1.1 ml min^{-1} flow rate). One microlitre of sample was injected splitless at 265°C. Temperature program was 40°C for 3 min, rising to 280°C at 5°C min^{-1}, held for 10 min. Compounds were identified by their mass spectra in a National Institute of Standards and Technology library search, and were confirmed by comparing the Kovats index with the indices of synthetic compounds.

(d) Electrophysiology

Gas chromatography coupled with electroantennographic detection (GC-EAD) and electroantennography (EAG) [25] were used to identify the antennal responses of the four sibling species to the collected *Pandanus* fruit volatiles. Flies were mounted following standard procedures [26]. For GC-EAD experiments, 1 µl of *Pandanus* extract or synthetic compound (*ca* 100 ng μl^{-1}, in DCM), was injected splitless into an Agilent GC 6890 equipped with a non-polar column (for details see above). A 1 : 1 effluent splitter allowed for the simultaneous flame ionization detection (FID) and the EAD of the separated compounds. Helium was the carrier

gas; injector and detector temperatures were 250°C and 300°C, respectively. Column temperature was held at 40°C for 1 min, rising to 300°C at 20°C min^{-1}, held for 10 min. The GC-separated components were introduced into a continuous, filtered and humidified air stream flowing over the antennae (1 l min^{-1}). The EAD and FID signals were simultaneously recorded and analysed (GcEad-1.2.0, Syntech, Hilversum, The Netherlands).

Determination of GC-EAD active compounds was simplified by converting the antennal responses into false-colour-coded heat maps using Fiji [27]. Therefore, EAD traces (exported in ASCII code) of single flies were imported into Fiji (as 'text image') and colour-coded ('smart'). Headspace compounds were determined as biologically active when eliciting reproducible responses in the fly antennae. EAD responses were manually quantified with the Syntech software [28], and normalized to the first external solvent-elicited peak.

Principal component analysis (PCA) was then applied (variance–covariance matrix), and the antennal responses were displayed within a three-dimensional space. With one-way analysis of similarity (ANOSIM), we tested by which degree the antennal responses of the four species differed from each other (Bray–Curtis similarity, sequential Bonferroni correction, 10 000 permutations). The similarity percentage (SIMPER) method [29] indicates compounds contributing to this dissimilarity [26]. Statistics were carried out with the software package PAST v. 2.11 [30].

For EAG experiments, synthetic compounds were diluted in mineral oil (Sigma-Aldrich, Steinheim, Germany) in decadic steps (10^{-1} to 10^{-5}) to record dose–response curves. *Prior to* each experiment, 10 µl of diluted odours was freshly loaded onto a small piece of filter paper (1 cm^2, Whatman, Dassel, Germany), and placed inside a glass Pasteur pipette. The antennae were held in a continuous, filtered and humidified air stream produced by a stimulus controller (Stimulus Controller CS-55, Syntech), whose flow was 1 l min^{-1}. Odour stimuli were applied for 0.5 s into the constant air stream. Antennal signals were recorded with EagProV2 (Syntech). Control stimuli consisted of filter paper with mineral oil, which was applied before each odours set. Response traces were baseline corrected with the mean of the first second and normalized to the control. Statistics (paired Student's *t*-test; one-way ANOVA with Turkey's post hoc test) were done with the software InStat v. 3.06 (GraphPad Software, Inc., San Diego, CA, USA).

(e) Functional imaging

To specify which glomerulus is activated by 3M2BA, we performed functional calcium imaging experiments as previously described [31]. Pure odorants were diluted (10^{-5}, 10^{-3}, 10^{-1}) in mineral oil (BioUltra, Sigma-Aldrich).

(f) Single sensillum recordings

To test the activation of the Or22a receptor by different 3M2BA concentrations, dose–response experiments were performed on the large basiconic sensilla in *D. erecta* and *D. melanogaster* females. Pure odorants were diluted (10^{-2}–10^{-8}) in mineral oil (BioUltra, Sigma-Aldrich). Single sensillum recording (SSR) measurements were performed as outlined in [32]. Responses to the solvent control were subtracted.

(g) Three-dimensional reconstruction of the antennal lobe

Anesthetized flies were dissected in *Drosophila* ringer solution as described by Wu & Luo [33]. The brains were fixed in 4 per cent PFA (4% paraformaldehyde, 0.1 M phosphate buffer, 0.2% Triton X-100) for 30 min on ice, and washed 3 × 20 min in PBST at room temperature (RT). Pre-incubation in PBST-NGS (PBST + 5% normal goat serum) lasted 1 h at RT. Brains were incubated in 1 : 30 mouse monoclonal nc82 antibody (the Developmental Studies Hybridoma Bank) in PBST-NGS, for 2 days at 4°C. After being washed 3 × 20 min at RT with PBST, brains were incubated in the secondary antibody, 1 : 200 goat anti-mouse Alexa Fluor 633 (Invitrogen, Darmstadt, Germany) in PBST-NGS, for 2 days at 4°C. Afterwards, brains were washed 3 × 20 min at RT with PBST, and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) on glass slides using spacers made of cover slides.

Whole-brain mounts were studied with a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Scans were performed for every 0.5 µm stack with a 40 × 10 water immersions objective (C-Apochromat 40x/1.2 W UV-Vis-NIR; Carl Zeiss). Structures labelled with Alexa Fluor 633 were excited with a HeNe laser at 633 nm.

Three-dimensional reconstructions and volumetric measurements of glomeruli were made with the segmentation software AMIRA v. 5.4.1 (Visage Imaging GmbH, Berlin, Germany). The ALs of three *D. melanogaster* and *D. erecta* females were analysed and compared. For each species, a template AL was chosen based on staining quality and shape. The terminology of the glomeruli is based on Couto [34]. Per species, the glomeruli volume was normalized to the total volume of the AL, which was equal in both species (*D. melanogaster* 63638.14 ± 3992.36 s.e.; *D. erecta* 68111.61 ± 3847.56 s.e.).

(h) Neuronal backfill

To trail the convergence of the OSNs of the ab3 sensilla, anterograde-neurobiotin backfills (Molecular Probes, Carlsbad, CA, USA) of *D. erecta* females were performed as previously described [5]. Treated flies were prepared for whole-brain mount confocal microscopy, as explained above (see §2*g*).

(i) Oviposition site preference

By using a two-choice assay, we tested the oviposition site preference (OSP) of the generalist *D. melanogaster* and the specialist *D. erecta* to 3M2BA. Tests were generally performed as previously described [32] with some modifications: indication was given that additional spatial information influences egg-laying behaviour in the flies. We, therefore, created homogeneous vertical structures by (i) gently separating the medium from the plate edges with help of a scalpel, and (ii) pressing the odour containing cup inside the medium. Experiments were conducted at 25°C, 12 L : 12 D photoperiod, 70 per cent relative humidity. Per species, we used six cages of 30 flies. Flies were allowed to oviposit for 40 h. For analyses, eggs were counted at three different positions (vertical medium edge, horizontal medium surface and vertical gap at the odour cup).

To test for the effect of odour and spatial information on the OSP, linear mixed effects models were performed using the computing environment R v. 2.15.2 [35]. Spatial and odour information were determined as fixed, cage identity and treatment served as random factors, with treatment nested in cage identity. The full model (with interaction between odour and position) was simplified by removing fixed factors step by step. Significance values for the fixed factors were obtained by comparing the models with a likelihood ratio test. *Prior to* the analyses numbers of eggs were log-transformed.

3. Results and Discussion

(a) Species-specific antennal detection of *Pandanus* volatiles does not reflect phylogeny

The purpose of this study was to unravel insect–host-derived adaptations within the olfactory system of *D. erecta* to its host, fresh fruits of the screw pine tree *Pandanus*. We, therefore,

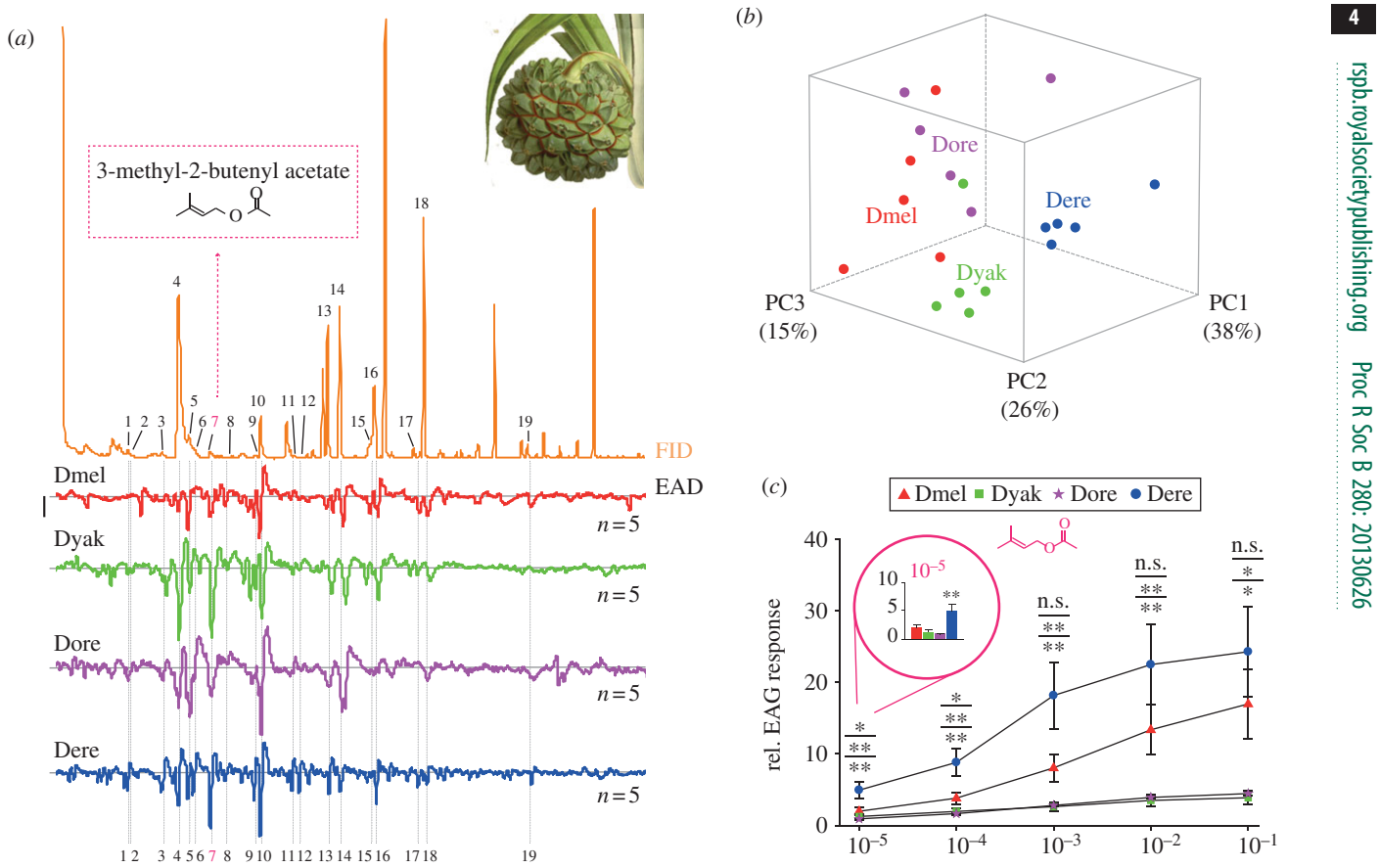

Figure 2. Antennal response spectra of the four *melanogaster* sibling species—evoked by the *Pandanus* sp. fruit headspace volatiles. (*a*) Headspace odour of *Pandanus* sp. fruits (upper part) and EAD responses (lower part). Bar stands for 1 mV of EAD response. Active compounds are coded as follows: 2,3-butanediol (no. 1), ethyl butyrate (no. 2), ethyl isovalerate (no. 3), isoamyl acetate (no. 4), 2-heptanone (no. 5), styrene (no. 6), 3M2BA (no. 7), ethyl tiglate (no. 8), 6-methyl-5-heptene-2one (no. 9), ethyl hexanoate (no. 10), linalool (no. 13), phenethyl alcohol (no. 14), benzyl acetate (no. 15), ethyl benzoate (no. 16), isopentyl hexanoate (no. 17), β-phenethyl acetate (no. 18). Peaks (no. 11), (no. 12) and (no. 19) are unidentified. Emphasized in pink is the novel *Drosophila* ligand 3M2BA (no. 7). (*b*) PCA (variance–covariance matrix) of quantified GC-EAD responses. PC1, 2 and 3 are plotted in three-dimensional space (79% cumulative variance). Species differ significantly from each other (one-way ANOSIM; Bray–Curtis distance; $R = 0.78$; $p < 0.001$). Isoamyl acetate (no. 4), 3M2BA (no. 7) and phenethyl alcohol (no. 14) most supported to the observed dissimilarity (SIMPER; all groups pooled; cumulative contribution 30.8%). (*c*) EAG dose–response curves of 3M2BA from the four species. *Drosophila erecta* is highly sensitive to 3M2BA, starting at 10^{-5} (paired *t*-test, $^{**}p < 0.01$, emphasized). *Drosophila erecta* differs from its siblings (one-way ANOVA; Turkey's post hoc test; $p > 0.05$ n.s.; $^{*}p < 0.05$; $^{**}p < 0.01$). Mean ± s.e. In all graphs, species abbreviation and colour-code is as follows: *D. melanogaster* (Dmel, red), *D. yakuba* (Dyak, green), *D. orena* (Dore, violet) and *D. erecta* (Dere, blue), and $n = 5$ per species. *Pandanus candelabrum* image adapted from an original illustration. Reproduced with the kind permission of the Director and the Board of Trustees, Royal Botanic Gardens, Kew.

followed a comparative approach using electrophysiology in four sympatric *melanogaster* sibling species (*D. melanogaster*, *D. yakuba*, *D. erecta* and *D. orena*; figure 1) with different host specificity and ecology (in the case of *D. orena*, with unknown ecology). First, we sampled volatiles from fresh *Pandanus* sp. fruits. The collected headspace was then examined for antennal activity in linked GC-EAD experiments [25]. Performing GC-EAD experiments with *D. melanogaster*, *D. yakuba*, *D. orena* and *D. erecta* as natural odorant detectors, we found a total of 19 volatiles eliciting reproducible antennal responses among the *Pandanus*-emitted volatiles (see figure 2*a* and electronic supplementary material, figure S1). We identified the biologically active compounds via GC-MS, through library comparison and co-injection of synthetic standards. We succeeded in identifying all but three minor peaks (nos 11, 12 and 19).

Notably, the *Pandanus* bouquet evoked species-specific response spectra on the fly antenna. However, only few and typically fruit-related compounds [36] induced very strong responses in all species (see figure 2*a* and electronic supplementary material, figure S1). We next asked whether the antennal response spectra towards the *Pandanus* bouquet varied between the four different species. First, we examined if the antennal response spectra simply mirrored the phylogenetic relationship between these species. To address this question, we performed a PCA based on the quantitative EAD responses to the 19 active peaks in the *Pandanus* headspace, and plotted the results within three-dimensional space (figure 2*b*). The *D. erecta* measurements formed a distinct cluster, which did not overlap with *D. orena*, its closest relative. Instead, *D. erecta* grouped closer with *D. yakuba*, a species also attracted to *Pandanus* syncarps [22]. On the other hand, *D. orena* clustered closer to the generalist *D. melanogaster*. The PCA, hence indicates that the generalist *D. yakuba* and the specialist *D. erecta* show similarities in their antennal response spectra, perhaps owing to the common food source [14]. The distinct clustering of the four species was mainly based on differential EAG responses to the three compounds, isoamyl acetate (no. 4), 3M2BA (no. 7) and phenethyl alcohol (no. 14; figure 2*a*). We thus conclude that the recorded olfactory responses of the different fly species probably reflect lifestyle rather than phylogenetic relationship.

(b) *Drosophila erecta* displays increased antennal sensitivity towards a key *Pandanus* volatile

Whereas, isoamyl acetate and phenethyl alcohol are common natural fruit ligands [36], 3M2BA attracted our particular interest. 3M2BA is fairly rare in nature, but has previously been reported as a diagnostic volatile from *Pandanus* syncarps [37]. Although 3M2BA was a relatively small constituent of the *Pandanus* fruit examined here, it evoked strong antennal responses in the GC-EAD experiments (figure 2*a*). Accordingly, we next examined the sensitivity of the four species towards this novel *Drosophila* odour ligand in more detail. In dose–response experiments, we recorded electroantennogram (EAG) activity from the four species, using synthetic 3M2BA as stimulus. The specialist *D. erecta* was considerably more sensitive than its relatives towards this *Pandanus* volatile (figure 2*c*). Our findings thus parallel earlier studies from other insects demonstrating increased sensitivity of specialist species towards host-specific volatiles [38,39]. In short, we assume that the increased sensitivity of *D. erecta* to 3M2BA represents a host-specific adaptation towards use of *Pandanus* syncarps. Next, we wondered which olfactory receptors (ORs) are activated by this ligand and where the respective OSNs target in the brain.

(c) 3-methyl-2-butenyl acetate predominantly activates ab3A type olfactory sensory neurons

To identify OR(s) activated by 3M2BA, we next performed functional imaging of the AL. Briefly, the AL consists of subunits, the so-called glomeruli, which are formed by afferents of the OSNs; these in turn are housed in the sensilla that cover the insect antenna. OSNs expressing the same receptor converge onto one specific glomerulus [40]. Because transgenic *D. erecta* are still not available, we performed these experiments with *D. melanogaster* females expressing the Ca^{2+} sensitive reporter *GCaMP3.0* [24] from the *Orco* promoter to get an indication of which glomeruli and, consequently, which ORs were activated by 3M2BA. Stimulation with diagnostic odours and comparison with the map of the fly AL [34,41] enabled us to identify activated glomeruli (figure 3*a–c*). 3M2BA activated three glomeruli, DM2, DM5 and DM6, of which DM2 showed the strongest response (figure 3*a*). In *D. melanogaster*, DM2 receives input from ab3A type neurons that express the olfactory receptor Or22a [42]. This neuron type detects fruity esters in *D. melanogaster* [36] as well as in the other species of the *melanogaster* species group, including *D. erecta*. The signals measured from the DM5 and DM6 glomeruli probably stem from light scattering from the strongly activated neighbouring DM2 glomerulus.

To verify that ab3A OSNs in *D. erecta* underlie the responsiveness towards 3M2BA, we next performed SSR from the three large basiconic sensilla in this species. Indeed, these OSNs respond to 3M2BA in *D. erecta* (figure 3*d*). In addition, we also noted responses from the ab2A neuron towards 3M2BA, albeit only at high concentrations. Dose–response experiments performed from OSNs in ab2 and ab3 sensilla also demonstrate the high sensitivity of ab3A neurons to 3M2BA (figure 3*e*). We note that in the functional imaging experiments, no significant increased activity was observed from the DM4. This discrepancy is probably owing to the low sensitivity of the ab2A OSNs towards this substance, making it difficult to observe the signal with conventional functional imaging. In line with the notion that the signals from the DM5 stem from light scattering from DM2, we also recorded no increased spike firing in response to stimulation with 3M2BA from the ab2B OSNs, whose axons target DM5.

Interestingly, the specialization of *D. erecta* towards *Pandanus* thus appears to involve the same pathway as affected in *D. sechellia*. In *D. erecta*, the ab3A/DM2 pathway mediates information regarding the *Pandanus* volatile 3M2BA, whereas in *D. sechellia* the homologous pathway handles the major noni volatile methyl hexanoate. In *D. sechellia*, the increased sensitivity to the noni volatile is also accompanied by morphological alterations within the AL. Hence, we wondered if we would also find similar modifications in *D. erecta*.

(d) Adaptation is mirrored in the internal and external olfactory organs

To identify potential changes in the morphology of the olfactory system, we next reconstructed ALs of *D. erecta*, and compared these with the well-established AL structure of *D. melanogaster* (figure 4*a*). The AL of *D. erecta* revealed a complex of four enlarged glomeruli at the medial lateral site. Comparing these with the AL atlas of *D. melanogaster* [34,41,43], we identified these as homologous to DM2, DM4, DM5 and VM5d. The relative volume of all glomeruli was enlarged up to 2.5 times in *D. erecta*, compared with *D. melanogaster* (DM2 × 1.73; DM4 × 1.71; DM5 × 1.6; VM5d × 2.5; figure 4*a*(ii)).

The main factor contributing to the increase in glomerular volume is probably enhanced antennal input, i.e. a higher number of a certain sensilla [3]. In the *D. erecta* AL, the most enlarged glomeruli (DM2 and VM5d), presumably both receive input from OSNs housed in ab3 type sensilla. Indeed, using data from a previous study [4], we estimated the number of large basiconic sensilla for the species under investigation, and found a higher percentage of ab3 type sensilla in *D. erecta* compared with the other species (figure 4*c*). The increased volume of both DM4 and DM5, which receive input from OSNs housed in ab2 type sensilla can also be explained by a proportional increase of these sensilla in *D. erecta*. The larger spiking neuron in this sensillum type, which targets the DM4 glomerulus, also responded to 3M2BA, albeit only at high concentrations.

To further verify that the enlarged glomeruli in *D. erecta* receive input from ab3 type sensilla, we next labelled OSNs via anterograde-neurobiotin backfills, allowing us to connect physiological response with the neuronal correlates in the AL [3]. Indeed, the 3M2BA sensitive OSNs housed in ab3 type sensilla of *D. erecta* target the enlarged glomeruli (figure 4*b*).

In short, adaptations towards use of *Pandanus* in *D. erecta* parallel to a striking degree those found for the noni-specialist *D. sechellia*, even involving the same subpopulation of OSNs and receptor genes. However, some distinctions have to be emphasized here. First, in *D. erecta*, unlike *D. sechellia*, increased sensitivity is not based on the complete replacement of another type of sensilla (figure 4*c*). Second, while ab3A neurons in *D. erecta* are increased in numbers, the homologous neurons in *D. sechellia* also display a change in sensitivity (figure 3*e*).

(e) 3-methyl-2-butenyl acetate triggers egg-laying in *Drosophila erecta*, but not in *Drosophila melanogaster*

Having unveiled the olfactory detection of host-derived signals in *D. erecta* females, we next examined the behavioural relevance of 3M2BA. Egg laying in *Drosophila* is a behaviour

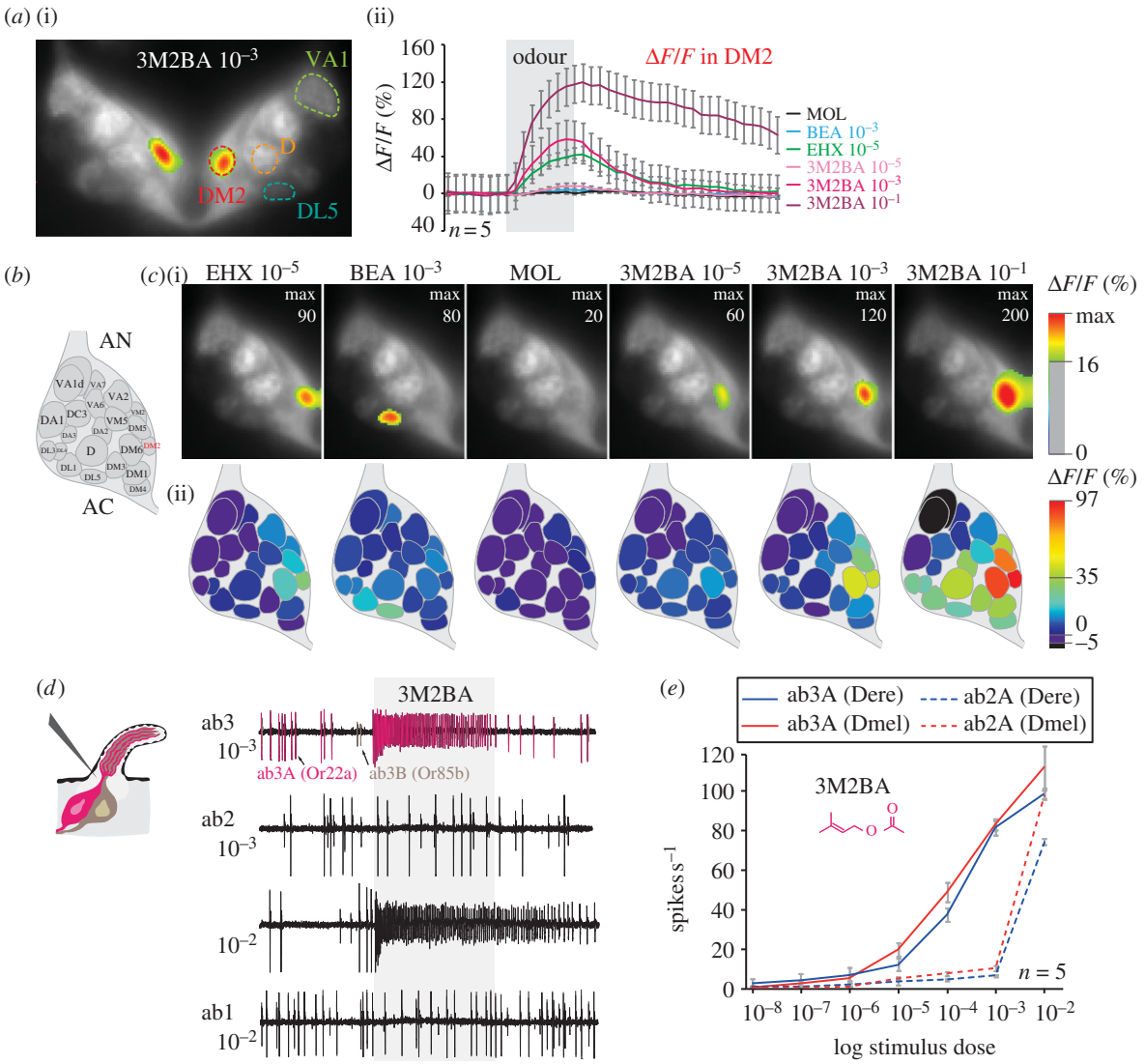

Figure 3. Identification of ORs activated by 3-methyl-2-butenyl acetate via functional imaging and single sensillum recordings. (*a*) Functional imaging in *D. melanogaster*. Representative recording of both ALs performed with 3M2BA (i), and activity traces of the DM2 glomerulus (ii) in response to the same set of odours as used in (*c*). Error bars represent s.d. (*b*) Glomerular atlas of the AL. (*c*) Representative recordings performed with different reference stimuli (EHX, ethyl hexanoate; BEA, benzaldehyde; and MOL, mineral oil) and different 3M2BA concentrations illustrate the strong activation of DM2 glomerulus by 3M2BA (i). Images are individually scaled to the strongest activated glomeruli. Values below the $\Delta F/F$ threshold are omitted to illustrate the specificity of the signals, as well as the glomerular arrangement as visualized by the intrinsic fluorescence. Corresponding odour-induced activity (average % $\Delta F/F$) plotted on schematic ALs (ii). (*d*) Single sensillum recording (SSR) measurements of the large basiconic sensilla in female *D. erecta* illustrate strong activation of the Or22a receptor (ab3A neuron) by 3M2BA. At higher concentrations, Or59b (ab2A neuron) is also activated. (*e*) SSR dose–response experiments performed on ab2 and ab3 sensilla in *D. erecta* (blue: solid line, ab3A Dere; dashed line, ab2A Dere) and *D. melanogaster* (red: solid line, ab3A Dmel; dashed line, ab2A Dmel) females with 3M2BA. Mean ± s.e.

that is under strong selection pressure [44]. We, therefore, tested the specialist *D. erecta* and generalist *D. melanogaster* within a two-choice oviposition experiment by offering them either a control or 3M2BA (figure 4*d*). There are indications that flies preferentially lay eggs along vertical structures [45,46]. We, therefore, implemented spatial information, in addition to odour information, into our experimental set-up.

Considering the effect of spatial information, both *D. erecta* and *D. melanogaster* clearly preferred the vertical medium surface over the vertical gap around the odour cup, and over the horizontal medium surface (*D. erecta*, $p = 0.003$; *D. melanogaster*, $p = 0.002$; figure 4*d*; solid blue bars). However, 3M2BA only triggered oviposition in *D. erecta*, but not in *D. melanogaster* (*D. erecta*, $p = 0.003$; *D. melanogaster*, $p > 0.05$; figure 4*d*; transparent bars). More strikingly, this effect was strongly enhanced when combined with spatial information (vertical structures). In combination with 3M2BA, *D. erecta* laid significantly more eggs inside the vertical gap around the odour cup than along the vertical medium surface (*D. erecta*, $p < 0.001$; *D. melanogaster*, $p > 0.05$; figure 4*d*; solid pink bars).

Preferring vertical edges for egg-laying might be advantageous for flies in general, because it may provide protection from predators and desiccation. It is conceivable that both odour and spatial information are needed to indicate reliable egg-laying places. The potential role of 3M2BA as the sole stimulus triggering oviposition preference for *Pandanus* in *D. erecta* is unlikely. Most probably, it is a combination of *Pandanus* volatiles that guides gravid females to the fruits and induces egg-laying. However, an enhanced sensitivity towards a host odour with limited availability outside of the host, such as 3M2BA, would be evolutionary beneficial.

4. Conclusion

Finding an appropriate breeding and feeding site is a crucial aspect in an insect's life, because the success of that choice

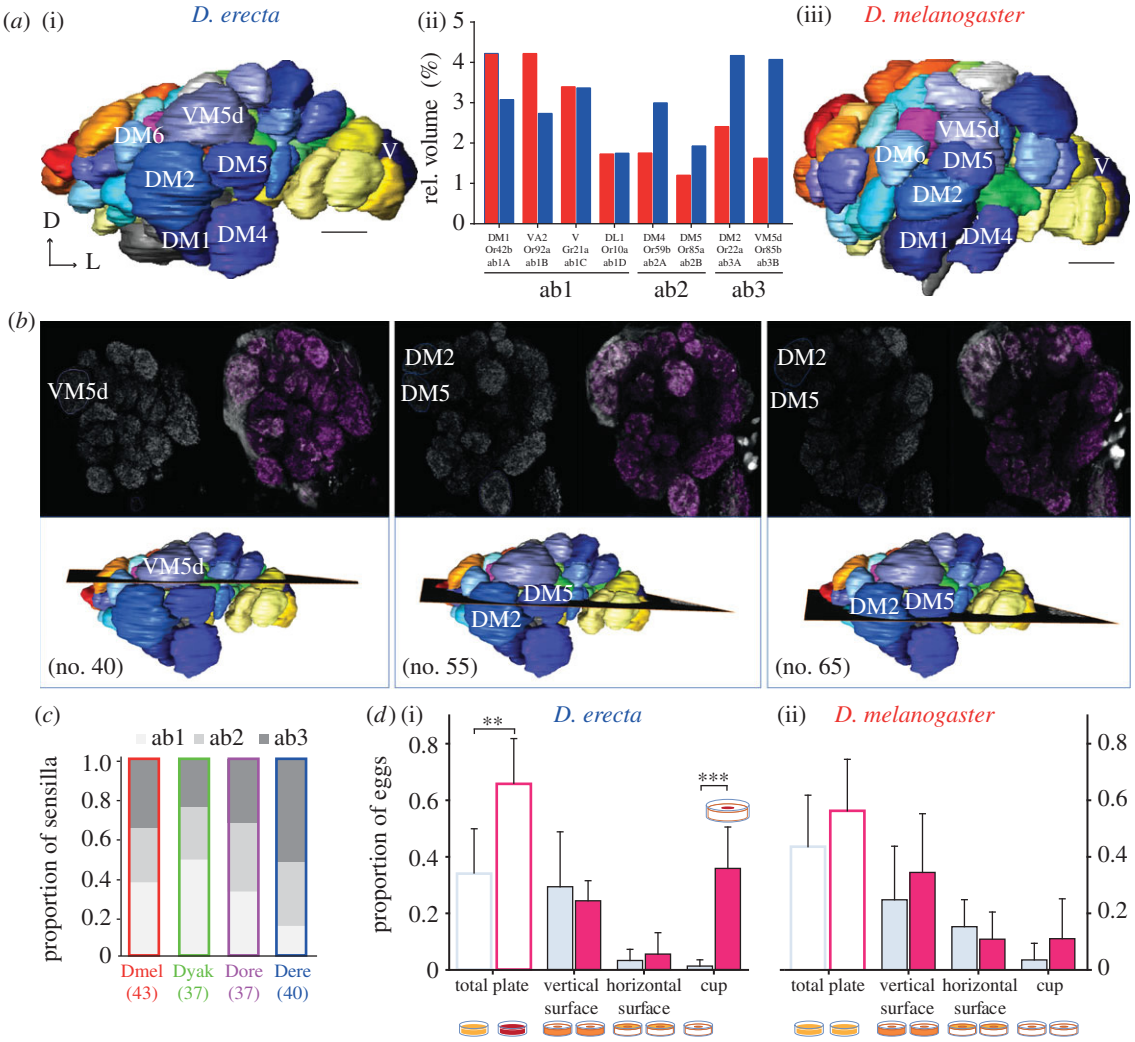

Figure 4. Morphological changes towards a complex of enlarged glomeruli. (*a*) Reconstruction of female antennal lobes (ALs) of the specialist *D. erecta* (i) and the generalist *D. melanogaster* (iii). Glomeruli terminology according to Couto [34]. ALs are viewed from medial to lateral. Comparison of the relative volume of the complex of enlarged glomeruli (based on the corresponding antennal input—large basiconic sensilla type ab1, ab2 and ab3) in *D. erecta* (blue), and *D. melanogaster* (red) (ii). The four glomeruli were up to 2.5 times enlarged in *D. erecta*, compared to the morphological structures in *D. melanogaster* (DM2 × 1.73; DM4 × 1.71; DM5 × 1.6; VM5d × 2.5). $n = 3$ for *D. melanogaster*, $n = 4$ for *D. erecta*. Scale bar = 10 µm. Dorsal (D), lateral (L). (*b*) Neuronal backfill of ab3 sensilla in female *D. erecta*, viewed in three different planes of the AL. Labelled axons converge into the region of enlarged glomeruli (upper part). The corresponding planes are displayed in the reconstructed AL. Numbers in the paranthesis correspond to the plane (lower part). (*c*) Relative number of large basiconic sensilla of the four species under investigation [4]. Species name abbreviations according to the first three species letters. Total numbers of sensilla are given in parentheses. (*d*) Influence of 3M2BA in combination with spatial information (vertical structures) in oviposition site preference in the specialist *D. erecta* (i) and the generalist *D. melanogaster* (ii). Transparent bars represent relative number of eggs counted on the plates in total (control, light blue; 3M2BA, pink); solid bars include spatial information (vertical surface; horizontal surface; and vertical surface around odour cup). Mean ± s.d. Spatial preference of *D. erecta* and *D. melanogaster*: vertical medium surface > vertical gap around the odour cup > horizontal medium surface (*D. erecta*, $p = 0.003$; *D. melanogaster*, $p = 0.002$). 3M2BA significantly triggered oviposition in *D. erecta*, but not in *D. melanogaster* (*D. erecta*, $p = 0.003$; *D. melanogaster*, $p > 0.05$). Combination 3M2BA and spatial: *D. erecta* laid significantly more eggs inside the vertical gap around the odour cup > vertical medium structure (*D. erecta*, $p < 0.001$; *D. melanogaster*, $p > 0.05$). Per species, $n = 6$ cage of 30 flies.

directly influences the offspring's nutritional intake and consequential fitness. In this regard, the sense of smell is critically important for many insects. Here, we studied the olfactory system of the specialist *D. erecta*, and provide evidence for adaptational changes in the insect towards its host and oviposition site, fruits of *Pandanus*. We not only found *D. erecta* being more sensitive towards host volatiles than its non-specialist siblings, we also demonstrate that the antennal response spectrum displays the life history rather than the phylogenetic relationship of the four flies investigated in this study. The higher sensitivity in *D. erecta* towards host volatiles is accompanied by changes in the AL morphology, specifically, a complex of enlarged glomeruli. We further uncovered the glomerular activation pattern and the receptor activation of 3M2BA, a novel *Drosophila* fruit ligand, which seems to play an important role in OSP of *D. erecta*.

In conclusion, olfactory adaptations in *D. erecta* appear to have occurred at two levels: first, at the periphery, with an increased number of ab3 sensilla; and second (as direct consequence), a shift towards a complex of enlarged glomeruli in the AL. One of these glomeruli, the DM2, is involved in processing the *Pandanus* key compound 3M2BA. Our results thus not only support previous findings in the noni fruit specialist *D. sechellia*, but also provide evidence for a general pattern of olfactory adaptations in insect–host associations.

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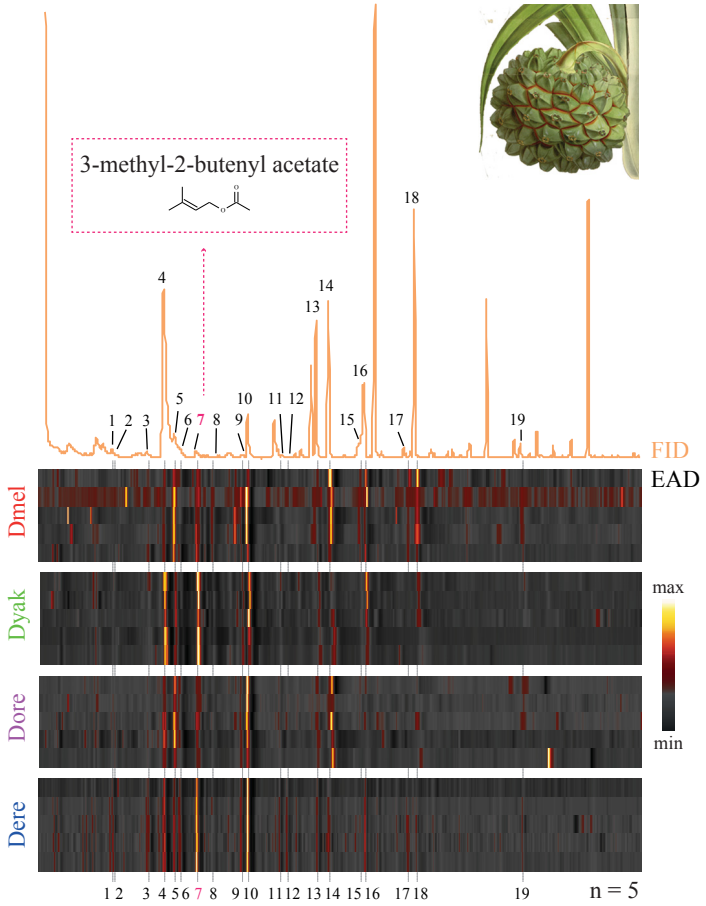
Supplemental 1



MANUSCRIPT IV

Divergence in Olfactory Host Plant Preference in *D. mojavensis* in Response to Cactus Host Use

Priya Date*, Hany K. M. Dweck*, Marcus C. Stensmyr, Jodi Shann, Bill S. Hansson, and Stephanie M. Rollmann

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Divergence in Olfactory Host Plant Preference in *D. mojavensis* in Response to Cactus Host Use

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Abstract

Divergence in host adaptive traits has been well studied from an ecological and evolutionary perspective, but identification of the proximate mechanisms underlying such divergence is less well understood. Behavioral preferences for host plants are often mediated by olfaction and shifts in preference may be accompanied by changes in the olfactory system. In this study, we examine the evolution of host plant preferences in cactophilic *Drosophila mojavensis* that feeds and breeds on different cacti throughout its range. We show divergence in electrophysiological responses and olfactory behavior among populations with host plant shifts. Specifically, significant divergence was observed in the Mojave Desert population that specializes on barrel cactus. Differences were observed in electrophysiological responses of the olfactory organs and in behavioral responses to barrel cactus volatiles. Together our results suggest that the peripheral nervous system has changed in response to different ecological environments and that these changes likely contribute to divergence among *D. mojavensis* populations.

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Introduction

Divergence of morphological, physiological, and behavioral traits as a result of local adaptation to different ecological environments is well documented [1]. Studies of host specialization in herbivorous insects, in particular, have been excellent models for understanding adaptive divergence in nature [2,3]. Conspecific populations can shift to alternate host plants, often because of changes in host plant availability. When such populations are geographically isolated, barriers to gene exchange can further contribute to divergence in host adaptive traits and ultimately may result in reproductive isolation among populations [4,5].

Understanding how reproductive isolation evolves requires an examination of the process prior to its completion [3]. Particularly promising are systems in which there is phenotypic divergence among populations of the same species from contrasting environments and for which extensive ecological data have been collected. *Drosophila mojavensis* represents such a system, and thus is a model of incipient speciation. *D. mojavensis* inhabits the arid regions of Baja California and the Sonoran and Mojave deserts of mainland Mexico and southern California and Arizona, respectively [6,7,8]. Changes in its range have been accompanied by changes in host plant use, with distinct populations of *D. mojavensis* using different cactus species across its range. The population in Baja California feeds and breeds on pitaya agria (*Stenocereus gummosus*), the mainland Sonoran/Arizona population uses organ pipe cactus (*S. thurberi*) and at times cina (*S. alamosensis*) cactus, and the populations in the Mojave Desert and on Santa Catalina Island utilize barrel (*Ferocactus cylindraceus*) and prickly pear cactus (*Opuntia* spp.), respectively [6,7] (Figure 1A). The Gulf of California acts as a geographic barrier, restricting gene flow between Baja California and mainland Sonoran populations [9]. These geographically isolated populations show differing levels of premating isolation but no postmating isolation from one another [8,9,10,11]. Its sibling species, *D. arizonae*, ranges from central Guatemala through mainland Mexico to Arizona, using columnar cacti and *Opuntia* hosts [8].

Drosophila mojavensis feeds and breeds on necrotic cactus tissue and the volatile compounds produced by the fermenting cactus are the primary sensory cue for host plant identification, and long range attraction to preferred oviposition sites [7,12]. Early studies of agria and organ pipe rot liquids suggests that host plant chemistry differs between cactus species in the composition and relative amounts of specific compounds [13,14]. Also, studies of behavioral preferences in *D. mojavensis* for agria and organ pipe rots, or for synthetic mixtures representing the composition of their liquid rots, suggest an overall preference for the agria host [12,13,15]. However, knowledge of the volatile compounds that form the odorant headspace surrounding any of the four host cacti is unknown. Moreover, the proximate mechanisms underlying differences in olfactory preferences in this species to host plant volatiles remains to be determined.

Here we examine the evolution of host plant specialization in *D. mojavensis*. We assess the volatile composition of fermenting cactus

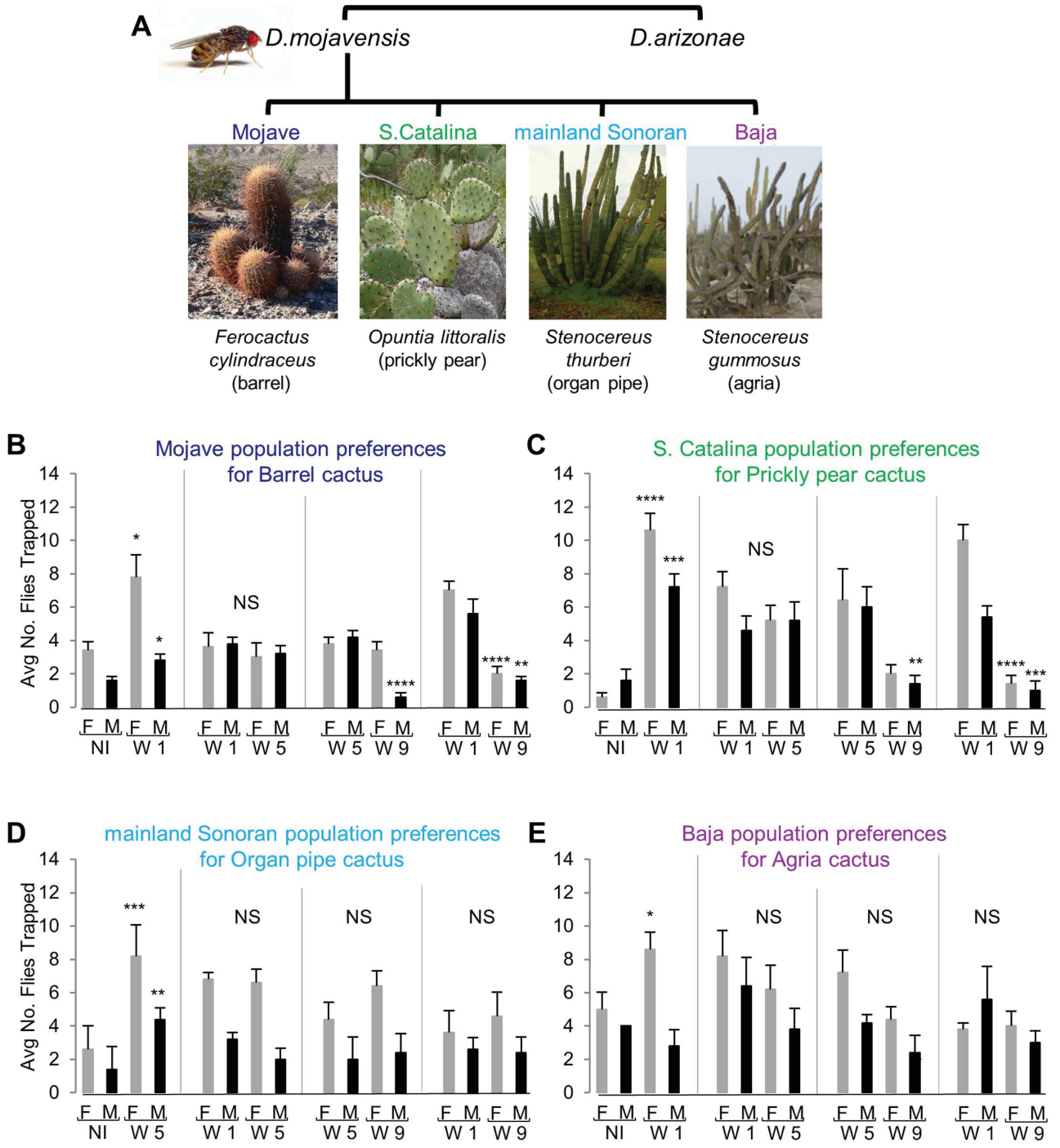

Figure 1. Changes in behavioral preferences with host plant fermentation stage. (**A**) *D. mojavensis* populations specialize on different host cacti across their range. Its sibling species, *D. arizonae* uses columnar cactus and *Opuntia* as hosts. (**B–E**) Two choice behavioral preferences of males (M) and females (F) of the Mojave, S. Catalina, mainland Sonoran, and Baja populations for their own respective host plants. Behavioral preferences for uninoculated (NI) host cactus in comparison to fermented host cactus and preferences for different stages of fermentation of a given host cactus are shown. Cactus tissues were fermented for one to nine weeks (W1– W9). For the mainland Sonoran population, comparisons between uninoculated and five week fermented organ pipe are shown as no significant difference between uninoculated and one week fermented organ pipe was found (data not shown). For panels **B–E**, behavioral preferences are shown as mean ± standard error and significance within a given sex and choice test is depicted by asterisks (*: $P<0.05$; ** $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$).
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tissues over time for all four host plants. We then test the hypothesis that adaptation to different host plant volatiles involves alterations at the sensory periphery by examining differences in electrophysiological responses of the olfactory organs among

populations. We measure behavioral responses of each population for different fermentation stages of their respective cacti and to specific cactus volatiles. Our findings begin to unravel the mechanisms underlying intraspecific divergence and the evolution of host-plant specialization in *D. mojavensis*.

Results

Behavioral Preferences for Cactus Fermentation Stage

The four populations of *D. mojavensis* feed and breed on four different species of fermenting cacti, so we began our study of the role of olfaction in host plant shift by measuring the attraction of each population to their respective host plants across a range of fermentation stages. The purpose of these experiments was to determine at what stage(s) the flies are attracted to their own host cactus necroses, an essential step in identifying the volatile(s) underlying host specific behavioral attraction. Thus, we conducted two choice experiments using a behavioral trap assay system. For each fly population the following comparisons were performed using their own respective host plants: uninoculated (NI) vs. one week (W1) fermented cactus, one week (W1) vs. five week (W5) fermented cactus, five week vs. nine week (W9) fermented cactus and lastly one week vs. nine week fermented cactus. For the mainland Sonoran population, comparisons between uninoculated and five week fermented cacti are shown as no significant difference between uninoculated and one week fermented samples was found (data not shown).

In these behavioral choice tests, all four populations overall showed greater attraction to fermented rather than fresh (uninoculated) cactus tissue (Figure 1B–E). Additionally, preferences in fermentation stage varied among the four populations. The Mojave Desert and S. Catalina populations had clear preferences for earlier fermentation stages (Figure 1B, C; Table S1A, B). Both one week and five week stages were equally attractive to flies, and attraction to nine week old tissue was reduced for both cacti. The mainland Sonoran and Baja populations, on the other hand, were attracted similarly to all of the stages of fermentation (Figure 1D, E; Table S1C, D). Moreover, increased attraction to fermented cactus in the mainland Sonoran population was only observed after five weeks of organ pipe fermentation, despite there being no difference in attractiveness between one and five week fermented samples (Figure 1D). There is also an indication of sex specific responses between some rot stage comparisons and overall females tended to have stronger responses. These results are expected given previous findings in *Drosophila* that show differences in olfactory responses between the sexes and increased behavioral responses in *D. mojavensis* females relative to males [12,16,17]. Finally, repetition of these behavioral tests using flies from a second fly line for each population lead to the same conclusion (data not shown), suggesting that changes in attraction with changes in headspace volatiles over time reflect a general population specific result.

Identification and Comparison of Volatile Composition Over Time

These differences in preference for different fermentation stages arise because volatile composition of fermenting cactus tissues is dynamic [18]. To determine changes in volatiles over time, we sampled the headspace of all four host cacti at one week intervals for nine weeks. Headspace volatiles varied between host plants and varied in their relative amounts across time (Figure 2A–D; Figure S1A–D). Seventy seven compounds were identified, with six unique to barrel, eight to prickly pear, two to agria and none to organ pipe (Table S2). More specifically, 1-dodecene, 2-methoxy-4-propyl phenol, durenol, isopropyl acetate, isopropyl propionate, and N,N'-diethyl-1,3 benzenediamine were unique to barrel cactus. The compounds 2-methyl-3-nonanol, 2-octanol acetate, ethyl propionate, isobutyl tiglate, isopropyl isopentanoate, isopropyl pentanoate, n-propyl 3-mercapto-propanoate, and pentanoic acid 1-methylpropyl ester were unique to prickly pear, and 6-methyl-2-heptanone and acetic acid were unique to agria. In general, the majority of identified compounds were esters (38%) and aromatics (30%). The volatile blends of prickly pear and agria were primarily equal in number of esters and aromatics, but organ pipe and barrel cacti were enriched for esters and aromatics, respectively. These host specific differences are illustrated by a principal component analysis (PCA) based on the volatile composition, in which the four host plants are clearly segregated into separate groups (Figure 2E; Table S3).

Electrophysiological Responses to Volatiles

Given that the volatile compositions of these host cacti differ substantially, we asked whether there was evidence of host specific adaptations in the olfactory systems of the fly populations. Intraspecific variation in odor-guided behavior has been observed previously in the tephritid fly *Rhagoletis pomonella* and these differences were accompanied by subtle changes in the peripheral odor detection machinery [19,20]. We examined whether there were alterations in the electrophysiological response properties of the antennae and maxillary palps using electroantennograms (EAG) and electropalpograms (EPG). We measured responses to 110 compounds for the antennae and 32 compounds for the maxillary palps among *D. mojavensis* populations and its sister species, *D. arizonae*. The odorants included diverse chemical groups as well as compounds present in fermenting host cacti ([13,14] and this study). Both EAG and EPG measurements indicated significant differences in odor detection, especially for the Mojave population (Figure 3A, B), as illustrated by PC analyses based on both EAG and EPG response characteristics. In both cases, the PCA grouped the *D. mojavensis* mainland Sonoran, Baja, and S. Catalina populations together with *D. arizonae*, and separate from the *D. mojavensis* Mojave population (EAG: ANOSIM based on Bray-Curts similarity, $R = 0.72$, $P < 0.0001$, Figure 3C; EPG: ANOSIM based on Bray-Curts similarity, $R = 0.625$, $P < 0.0001$, Figure 3D). Specifically, the antennae of the Mojave population differed primarily in having an overall reduced response to straight-chain esters, and the palps differed in having a strong response to 4-ethylguaiacol, a compound that elicited minimal responses from the other populations and *D. arizonae*. Moreover, as with rot preference behavior, electrophysiological responses did not differ significantly within populations (Table S1E). Therefore, these distinct odor sensitivities of the Mojave population presumably constitute host-specific adaptations. The headspace of barrel cacti, the sole host of the Mojave population, had a lower number of esters and those esters identified typically constitute only minor components of the volatile blend (see section on the identification and comparison of volatile composition over time, above). However, aromatics are a dominant component of barrel cactus headspace, with compounds such as 4-ethylguaiacol, present across all fermentation stages but present only in trace amounts in prickly pear and organ pipe and not detected in agria headspace (Table S2).

Behavioral Responses to Specific Host Plant Volatiles: Mixtures

Given the shifts in peripheral detection, we measured behavioral responses of the four *D. mojavensis* populations to a mixture of thirteen compounds for which the majority showed population

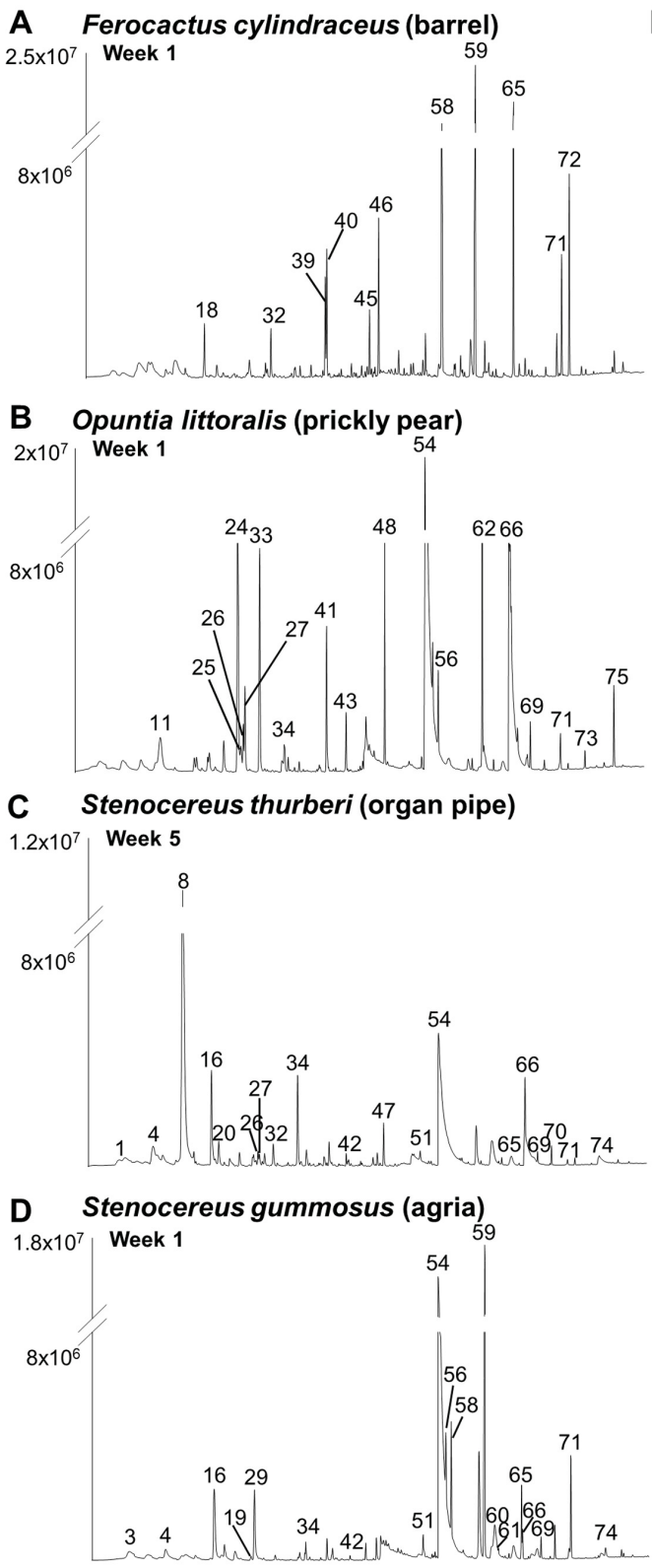

Figure 2. Typical gas chromatograms of headspace for the four cactus hosts. (**A–D**) Barrel, prickly pear, organ pipe and agria cactus headspaces, respectively, from fermented samples are shown. Peak numbers correspond to compounds identified in time course experiment presented in Figure S1. (**E**) Principal component (PC) analysis of the volatile samples from all four cacti. The eigenvectors for the PCs are provided in Table S3. The fifty percent density eclipses for the cacti are indicated with different line styles.
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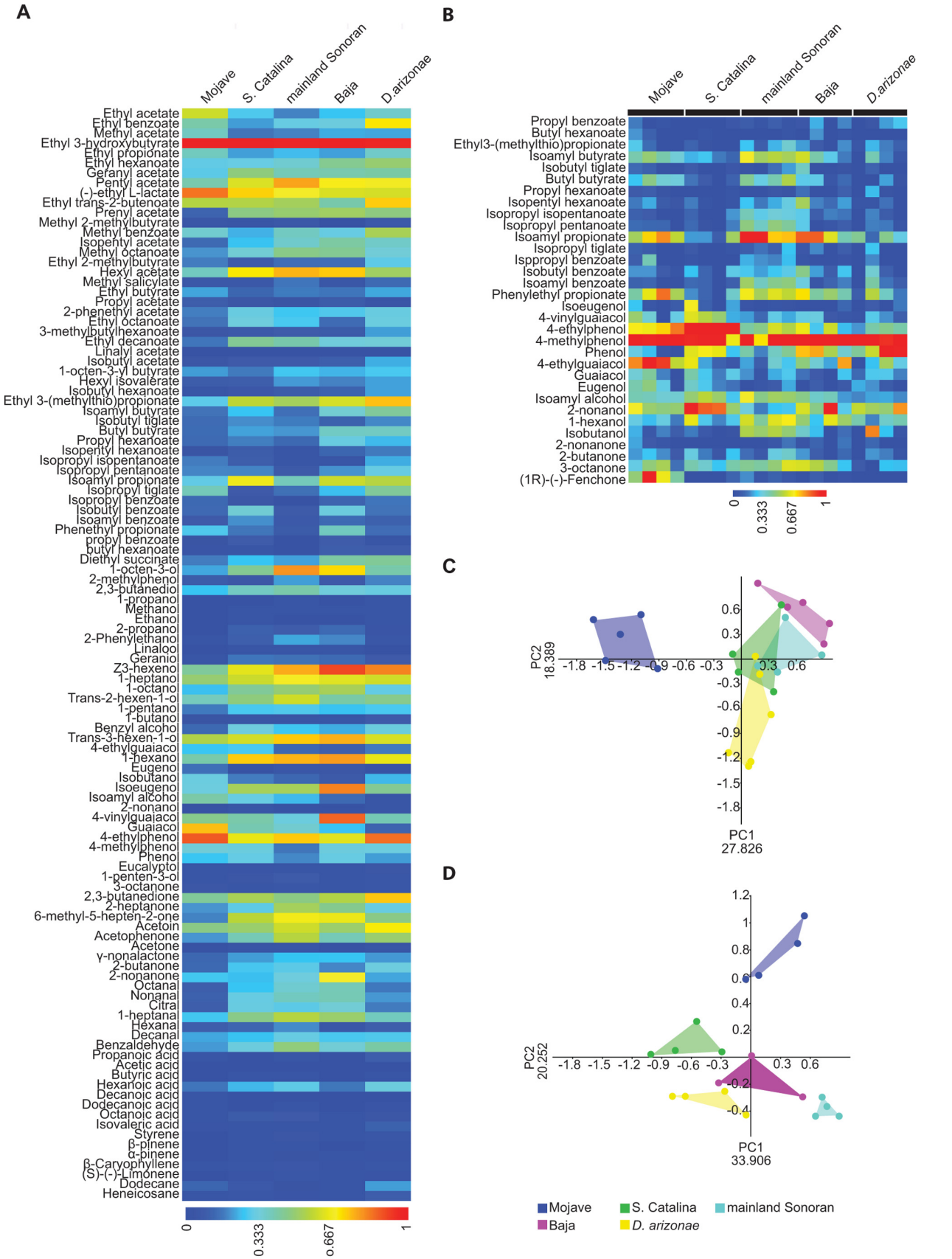
A
Mojave
S. Catalina
mainland Sonoran
Baja
D.arizonae
Ethyl acetate
Ethyl benzoate
Methyl acetate
Ethyl 3-hydroxybutyrate
Ethyl propionate
Ethyl hexanoate
Geranyl acetate
Pentyl acetate
(-)-ethyl L-lactate
Ethyl trans-2-butenoate
Prenyl acetate
Methyl 2-methylbutyrate
Methyl benzoate
Isopentyl acetate
Methyl octanoate
Ethyl 2-methylbutyrate
Hexyl acetate
Methyl salicylate
Ethyl butyrate
Propyl acetate
2-phenethyl acetate
Ethyl octanoate
3-methylbutylhexanoate
Ethyl decanoate
Linalyl acetate
Isobutyl acetate
1-octen-3-yl butyrate
Hexyl isovalerate
Isobutyl hexanoate
Ethyl 3-(methylthio)propionate
Isoamyl butyrate
Isobutyl tiglate
Butyl butyrate
Propyl hexanoate
Isopentyl hexanoate
Isopropyl isopentanoate
Isopropyl pentanoate
Isoamyl propionate
Isopropyl tiglate
Isopropyl benzoate
Isobutyl benzoate
Isoamyl benzoate
Phenethyl propionate
propyl benzoate
butyl hexanoate
Diethyl succinate
1-octen-3-ol
2-methylphenol
2,3-butanediol
1-propanol
Methanol
Ethanol
2-propanol
2-Phenylethanol
Linalool
Geraniol
Z3-hexenol
1-heptanol
1-octanol
Trans-2-hexen-1-ol
1-pentanol
1-butanol
Benzyl alcohol
Trans-3-hexen-1-ol
4-ethylguaiacol
1-hexanol
Eugenol
Isobutanol
Isoeugenol
Isoamyl alcohol
2-nonanol
4-vinylguaiacol
Guaiacol
4-ethylphenol
4-methylphenol
Phenol
Eucalyptol
1-penten-3-ol
3-octanone
2,3-butanedione
2-heptanone
6-methyl-5-hepten-2-one
Acetoin
Acetophenone
Acetone
γ-nonalactone
2-butanone
2-nonanone
Octanal
Nonanal
Citral
1-heptanal
Hexanal
Decanal
Benzaldehyde
Propanoic acid
Acetic acid
Butyric acid
Hexanoic acid
Decanoic acid
Dodecanoic acid
Octanoic acid
Isovaleric acid
Styrene
β-pinene
α-pinene
β-Caryophyllene
(S)-(-)-Limonene
Dodecane
Heneicosane
0
0.333
o.667
1
B
Mojave
S. Catalina
mainland Sonoran
Baja
D.arizonae
Propyl benzoate
Butyl hexanoate
Ethyl3-(methylthio)propionate
Isoamyl butyrate
Isobutyl tiglate
Butyl butyrate
Propyl hexanoate
Isopentyl hexanoate
Isopropyl isopentanoate
Isopropyl pentanoate
Isoamyl propionate
Isopropyl tiglate
Ispppropyl benzoate
Isobutyl benzoate
Isoamyl benzoate
Phenylethyl propionate
Isoeugenol
4-vinylguaiacol
4-ethylphenol
4-methylphenol
Phenol
4-ethylguaiacol
Guaiacol
Eugenol
Isoamyl alcohol
2-nonanol
1-hexanol
Isobutanol
2-nonanone
2-butanone
3-octanone
(1R)-(-)-Fenchone
0
0.333
0.667
1
C
PC2
18.389
PC1
27.826
D
PC2
20.252
PC1
33.906
Mojave
S. Catalina
mainland Sonoran
Baja
D. arizonae

Figure 3. Differences in electrophysiological response properties among the four *D. mojavensis* populations and *D. arizonae*. (**A–B**) Heat map of EAG and EPG responses (respectively) to a suite of odorants for *D. mojavensis* populations and *D. arizonae*. EAG and EPG responses were scaled to a range from 0 to 1. (**C–D**) PCA of EAG and EPG responses, respectively, to a suite of odorants for *D. mojavensis* populations and *D. arizonae*.
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differences in electrophysiological responses and/or were consistently present in barrel cactus. Since responses (electrophysiological and behavioral) did not differ significantly within populations, we focused our behavioral analyses of synthetic compounds on a single line per population. As expected, the Mojave population showed greater attraction to the mixture than the other *D. mojavensis* populations (Figure 4; Table S1F). Females in particular, showed attraction to the mixture in a dose dependent manner, which is consistent with previous studies [16,17] showing increased behavioral responses in females relative to males. Moreover, females of the Mojave population continued to show attraction to the mixture at a 10^{-2} dilution, while responses of females from the other three populations ranged from decreased attraction to repulsion. In the case of the Baja population, for example, dose responses were shifted to lower concentrations. We also tested responses to the thirteen compounds that were components of the mixture individually, at several concentrations (Figure 5; Table S1G). Most of the single compounds elicited minimal attraction or repulsion across all populations. Furthermore, those single compounds that elicited population specific differences did not recapitulate the host specific responses of the mixture, indicating that a combination of volatiles is essential for appropriate host plant identification and preference.

Discussion

Behavioral Responses to Host Plant Volatiles

We observed differences in olfactory preferences in *D. mojavensis* for different stages of host plant fermentation. These fermentation stages varied in the composition and abundance of volatiles produced. Early studies of the Baja population have shown that flies prefer fermenting cactus to fresh tissue and have an attraction to initial fermentation stages [13]. Our results are in accordance with these studies in that the Mojave and Catalina populations also exhibit a preference for early stages of cactus fermentation. No difference in preference among fermentation stages, however, was observed for the mainland Sonoran and Baja populations for their respective host plants. This lack of differential attraction was consistent with comparatively little change in the volatile compositions of these cacti over the test period and most likely reflect methodological differences between our study and Downing, 1985 [13]. Moreover, while rotting in nature likely occurs more rapidly and additional studies on microbe colonization of damaged cactus tissue in nature are needed [21], these laboratory experiments can identify volatiles and changes in volatiles that underlie shifts in host plant preference behavior.

Previous studies of single compounds have been instrumental in the development of our understanding of how olfactory cues are processed [22]. In nature, however, organisms encounter a vast array of volatiles and the importance of single compounds in an ecological context remains less clear. Our results show that individual compounds may elicit behavioral responses in *D. mojavensis*, but that host-specific attraction to a mixture of these compounds could not be explained by responses to a single compound alone. The importance of odor mixtures in mediating appropriate behavioral responses has been observed in other systems (e.g., grapevine moth (*Lobesia botrana*), [23]; oriental fruit moth (*Cydia molesta*), [24]; hawkmoth (*Manduca sexta*) [25]) and our results support a model in which synergistic or antagonistic effects among mixture compounds result in host specific behavioral responses to olfactory cues.

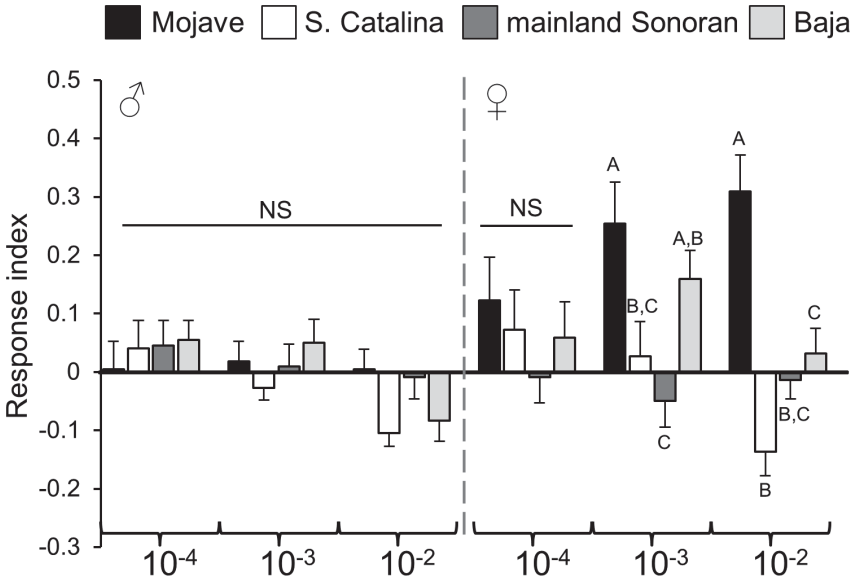

Figure 4. Host specific behavioral preferences for a synthetic mixture of 13 barrel cactus compounds. Behavioral responses of each population to the synthetic mixture were measured at mixture dilutions of 10^{-4}, 10^{-3}, and 10^{-2}. Response indices (mean ± std error) were calculated for each sex and *D. mojavensis* population. Comparisons among populations were made within a given mixture dilution and the letters above the bars denote significant differences in behavioral response among the populations (posthoc Tukey-Kramer test).
doi:10.1371/journal.pone.0070027.g004

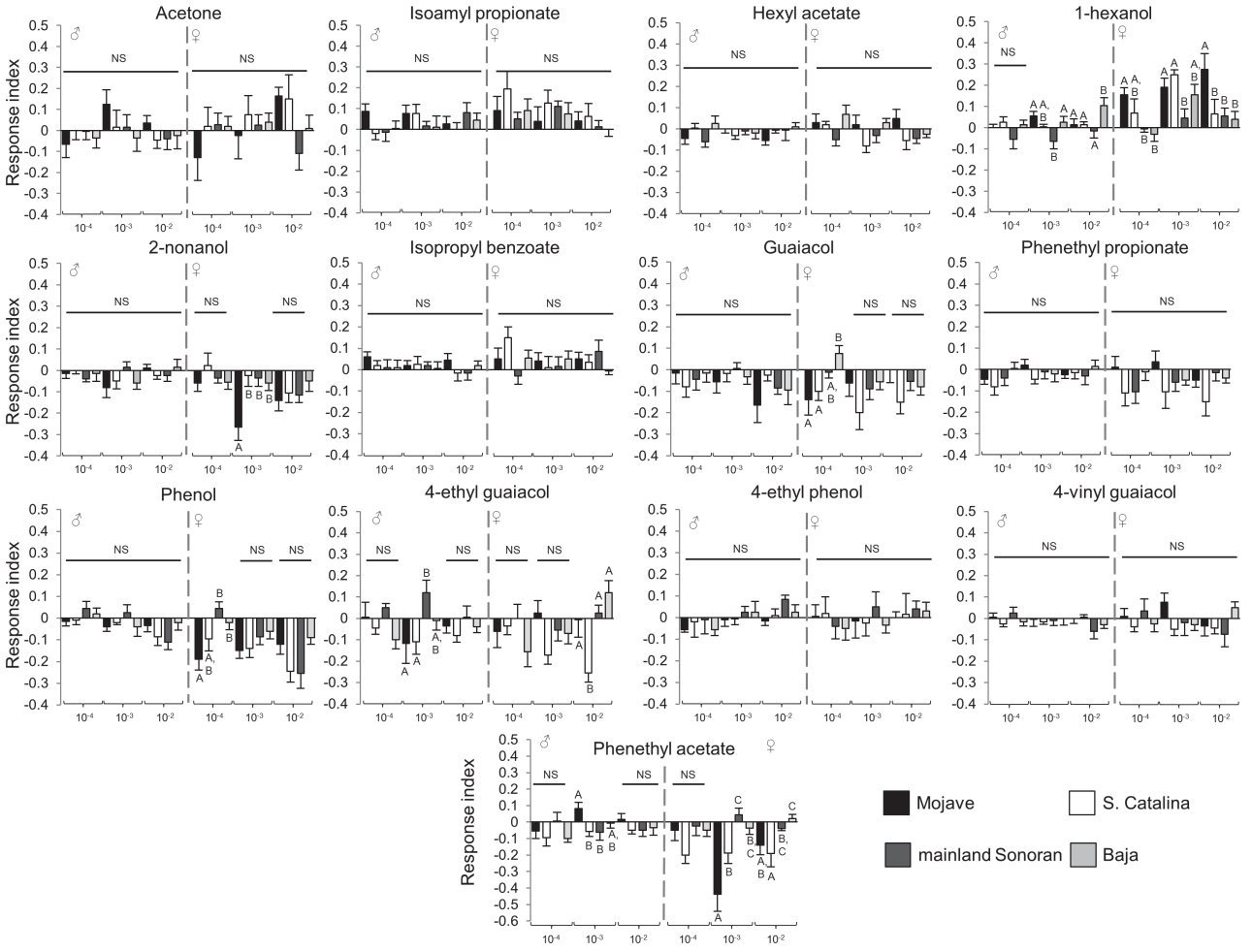

Figure 5. Behavioral responses to single compounds. Dose response indices (mean ± std error) of males (M) and females (F) for single compounds. Significant differences among *D. mojavensis* populations are denoted by different letters above the bars.
doi:10.1371/journal.pone.0070027.g005

Determinants of Olfactory Preferences

Adaptation to different ecological environments can result in divergence of olfactory preference [26]. We examined whether alterations at the sensory periphery were found among populations of *D. mojavensis* that differ in their host plant use. Notably we observed that the Mojave Desert population, specializing on barrel cactus, has diverged in its olfactory sensitivities with an overall decreased response to esters and increased response to aromatics. This divergence from the other three populations coincides with the fact that the volatiles released by fermenting barrel cactus are heavy in aromatics compared to the other three host cacti whose primary volatiles were enriched for esters or balanced equally with aromatics ([13,14] and this study). These electrophysiological differences most likely reflect alterations in ligand binding or odorant clearance at the sensory periphery, either through changes in gene expression or protein structure-function. The latter, caused by amino acid substitutions in chemosensory receptors, has been shown to confer differences in odorant sensitivity [27,28,29]. On the other hand, changes in the number of olfactory sensory neurons can also tune olfactory sensitivity towards host-specific volatiles, as in the case of *D. sechellia*, a specialist on *Morinda* fruit [30,31]. Moreover, this preference by *D. sechellia* for *Morinda* fruit volatiles has been shown to be mediated by odorant binding proteins [32] and host-driven sensory augmentation has been shown for other insects, such as *Culex* mosquitoes [33].

The Evolution of Olfactory Preference in *D. mojavensis*

In short, we have begun to understand the evolution of olfactory preference in response to host plant shift in *D. mojavensis*, a model of incipient speciation. Our results suggest rapid adaptation to changes in host plant utilization in this system. Estimates of divergence between.

D. mojavensis and *D. arizonae* range between 1.91 and 2.97 million years ago [34,35]. Moreover, Smith et al., 2012 [36] estimates that the Baja population diverged from an ancestral mainland Sonoran/Mojave Desert group 230,000 to 270,000 years ago. Separation of the mainland Sonoran and Mojave Desert populations are then estimated to have occurred 117,000 to 135,000 years ago. Such rapid adaptation of the olfactory system has also been observed in *Rhagoletis*, with shifts in olfactory preferences from hawthorn to apple within 150 years [37]. In the aforementioned studies of shift of host preference in *D. sechellia*, the shift was proposed to be in response to competition with *D. simulans* [38], with divergence between species less than half a million years ago [39]. Our findings in this system will help

unravel mechanisms underlying the process of species formation and the evolution host-plant specialization.

Materials and Methods

Identification of Host Plant Volatiles

An analysis of the volatile compositions of *Stenocereus gummosus*, *S. thurberi*, *Ferocactus cylindraceus*, and *Opuntia littoralis*, was obtained through headspace solid phase microextraction (SPME, Polydimethylsiloxane/Divinylbenzene, Sigma-Aldrich, St. Louis, MO). For each cactus, volatile compounds emitted from uninoculated and inoculated cactus were identified. The tissue was kept frozen and for the experiments it was thawed, placed in a glass jar with a polyethylene lined cap, sterilized and subsequently inoculated. More specifically, a 70 g piece of cactus tissue was inoculated with 1.0 ml of seven yeast species (*Pichia cactophila, P. mexicana, Starmera amethionina, Candida valida, C. sonorensis, Diapodascus starmeri and Sporopachyderma cereana*) mixture and 0.5 ml of one pectolytic bacterium *Erwinia cacticida* ([40,41] and Etges pers. comm.). Both yeast and bacteria cultures were freshly grown on Yeast Complete media or Glucose Yeast Calcium carbonate media plates respectively. After 48 hours, the microorganisms were harvested and suspended in sterile water. To facilitate the even distribution of the microorganisms, the cactus tissue was subsequently inoculated at multiple spots using a syringe [40,41]. These yeast species have been documented on necrotic cacti [18] and used previously in *D. mojavensis* rearing experiments [41]. Cactus tissue was then incubated at 30°C and the volatiles present in the headspace were determined at weekly intervals over nine weeks. The volatile compositions of two to three replicate rots per cactus were examined over the nine week period. The identification of volatiles emitted from uninoculated cactus tissue was determined after one day.

The SPME fiber was exposed for one hour to the sample headspace, and the fiber assembly was then placed into the GC-MS injector port. Volatiles were analyzed using an Agilent 7890A GC with 5975C MSD apparatus (Santa Clara, CA) in a pulsed splitless mode. The GC-MS was equipped with a polyethylene glycol column (Nukol, Supelco Co.). GC conditions were optimized with standards and subsequent analyses done at injector and detector (FID) temperatures of 250°C and 280°C, respectively. Helium was used as the carrier gas at 25 ml min^{-1}, and at a split ratio of 2:1. The oven temperature was initially set at 40°C for 1 min and then ramped to 210°C at a rate of 7° min^{-1}. Mass spectra were recorded from 35 to 700 amu, with electronic impact ionization at 70eV. Compounds were identified using the NIST Mass Spectral Library, by comparison to their retention times, and by mass spectra analyses of select standards. Compounds with more than a 90% match with the NIST library were labeled. Raw data was subjected to principal component analysis (PCA) using JMP 9.0 (SAS Institute, Cary, NC).

Drosophila Stocks

Flies were obtained from the Drosophila Species Stock Center or kindly provided by Dr. Bill Etges and are as follows: Baja California population, [Punta Prieta (stock number 15081–1351.30) and San Quintin (SQ59a)]; the mainland Sonoran population [Organ Pipe National Monument, Arizona, stock number 15081–1352.32 and OPNM9]; the Mojave population [Grand Canyon, Arizona (stock number 15081–1352.10) and Providence Mountain, CA (A997b)]; Santa Catalina Island [stock numbers 15081–1352.30 and 15081–1352.22]; *D. arizonae* [Sinaloa, Mexico (stock number 15081–1271.33)]. All flies were reared on cactus-banana-agar medium and were maintained at 25 °C, under a 12 h L/D cycle.

Electrophysiological Recordings

Odorants. Pure odorants were diluted (10^{-3}) in hexane or in water as appropriate. Diluted odors (10 µl) were pipetteted onto a small piece of filter paper (~1 cm^2) and placed inside a glass Pasteur pipette. For odorant application, a stimulus controller was used (Stimulus Controller CS-55, Syntech, Hilversum, The Netherlands). All odorants were obtained from Sigma-Aldrich (St. Louis, MO) at the highest purity available.

Electroantennograms/electropalpograms. Individual flies were immobilized in a pipette tip with the head partially protruding. Reference and recording glass capillary electrodes were filled with haemolymph Ringers. The reference electrode was inserted into one eye and the recording electrode brought into contact with either the proximal third antennal segment or the distal portion of the maxillary palps. A constant flow of charcoal-filtered and humidified air (1 l min^{-1}) was delivered at a velocity of 0.5 ms^{-1}, through a tube with its outlet approximately 10 mm from the antenna/palp. Odorant was introduced by placing the tip of the pipette through a hole in the side of this tube. The EAG signal (transferred via Ag-AgCl wires) was pre-amplified (10x) with a probe connected to a high-impedance DC-amplifier (EAG-probe Version2, Syntech) and digitally converted (IDAC-4 USB, Syntech), visualized and recorded on a PC using a dedicated software (EAG-probe, Syntech). Recordings were obtained from 2–4 individuals per sex and line. Traces of individual flies were scaled to a range from 0 to 1. Quantitative reactions to odor compounds were used for principal component analysis (via variance covariance). Calculations were done with PAST (http://folk.uio.no/ohammer/past/download.html) and SPSS software Version 17 (SPSS, www. Spss.com). To assess the degree of similarity between lines within a population, electrophysiological responses were measured to eleven odorants for lines of the Mojave population and the S. Catalina population. The odorants were selected based on their ability to elicit a range of responses.

Behavioral Trap Assay

Free walking behavioral assays consisted of twenty flies placed into a polystyrene arena (6 cm (H) ×15 cm (Ø)) containing two traps. Each trap was constructed using a 10 ml glass beaker, fitted with a polypropylene plastic funnel. Traps were then symmetrically placed within the testing arena. To prevent dehydration of the flies, a cotton ball saturated with 20 ml of water was placed into the arena. Flies were tested at 10–12 days post-eclosion and flies were starved overnight prior to the experiment. Assays were performed in the dark and the number of flies trapped was recorded after 48 hours. For tests of single odorants or synthetic mixtures, traps contained 2 ml of the vehicle control and 0.1% Triton X with or without odorant(s). All odorants were obtained from Sigma-Aldrich (St. Louis, MO) at the highest purity available. Behavioral responses were measured to a synthetic mixture of 13 individual compounds at proportions reflective of the headspace of a three week fermented barrel cactus (Table S2). These mixture included hexyl acetate, acetone, phenethyl acetate, guaiacol, 1-hexanol, 2-nonanol, 4-ethyl guaiacol, phenol, isopropyl benzoate, phenethyl propionate, isoamyl propionate, 4-ethyl phenol, and 4-vinyl guaiacol. Because 4-vinyl guaiacol was present in the majority of early stage rots, with the exception of week 3, it was included at its average relative amount across weeks one through five. All mixture components were also tested singly. Response indices were calculated by subtracting the number of flies present in the control traps from the number of flies present in

the trap containing odor and dividing by the total number of flies. Ten replicate measurements per sex, population and odorant concentration were conducted. Statistical analyses were conducted using ANOVA, followed by a Tukey-Kramer post-hoc test. For behavioral tests using fermenting cactus, all four cacti were inoculated as described in the above identification of host plant volatile section. Two grams of uninoculated or fermented cactus tissue was used per trap. Five replicate measurements per sex and per choice test were conducted. Statistical analyses were conducted within a given sex and two choice test using ANOVA. All analyses were done using JMP 9.0 software (SAS Institute, Cary, NC).

Supporting Information

Figure S1 Analysis of host plant volatile composition with cactus rot stage. Cacti were either uninoculated (NI) or inoculated and fermented for one to nine weeks. Peak numbers correspond to the list of volatiles. **(A–D)** Typical gas chromatograms of barrel, prickly pear, organ pipe and agria headspace (respectively) from uninoculated or representative fermented samples (weeks 1, 5, and 9).
(PDF)

Table S1 Analysis of variance for all experiments. **(A–D)** Experiments testing preference for different fermentation stages of barrel, prickly pear, organ pipe and agria cacti, respectively. **(E)** Comparisons of electrophysiological responses between lines within a *D. mojavensis* population. **(F)** Behavioral responses to the synthetic mixture. **(G)** Behavioral responses of single compounds.
(PDF)

Table S2 Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti.
(PDF)

Table S3 Principal component values for volatile compounds in the four host cacti. Eigenvectors with highest scores are indicated in bold. The compounds which were present only once across all four cacti were excluded from the PCA.
(PDF)

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Author Contributions

Conceived and designed the experiments: PD HKMD MCS JS BSH SMR. Performed the experiments: PD HKMD. Analyzed the data: PD HKMD MCS JS BSH SMR. Contributed reagents/materials/analysis tools: MCS JS BSH SMR. Wrote the paper: PD HKMD MCS SMR.

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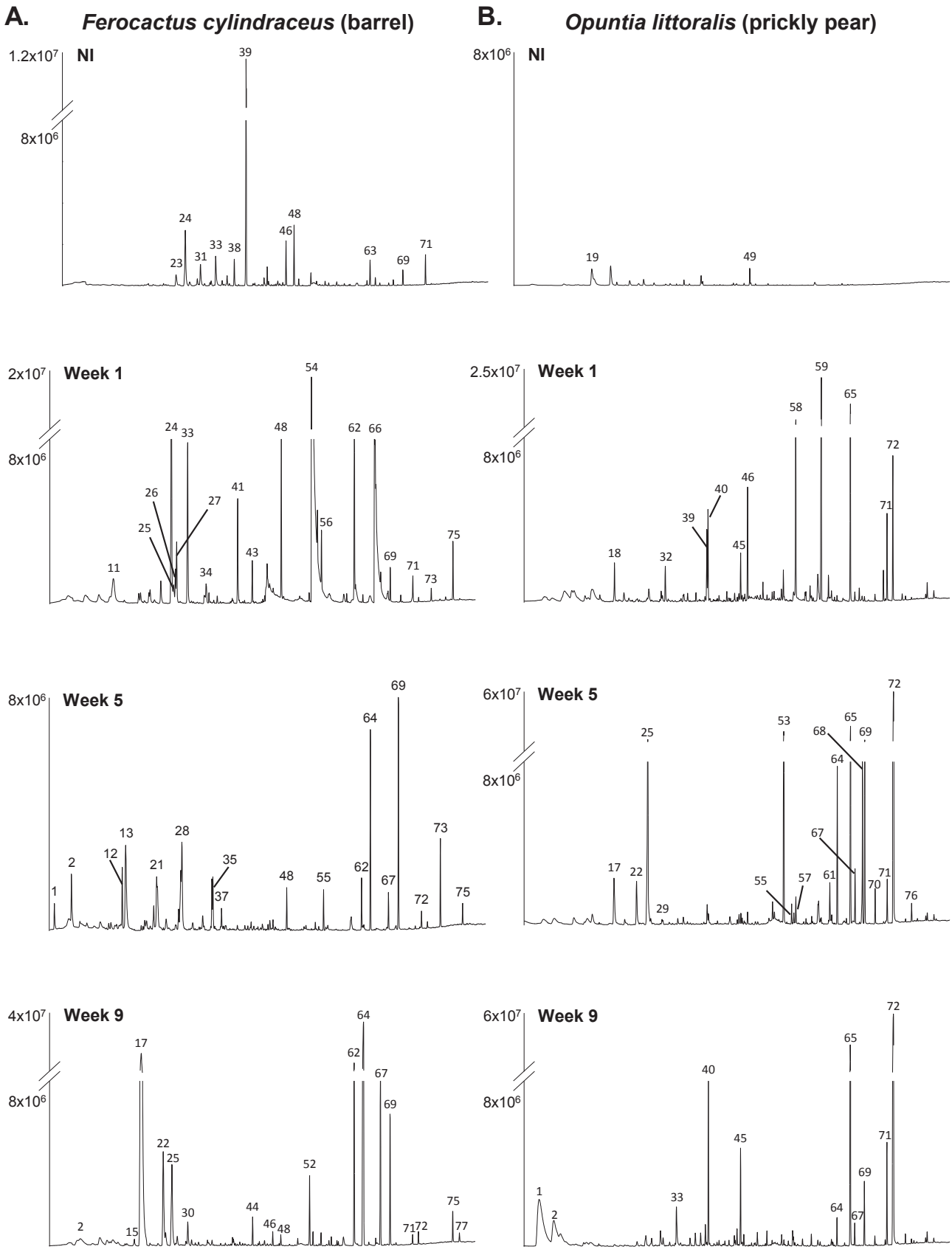
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Ferocactus cylindraceus (barrel)
B.
Opuntia littoralis (prickly pear)
NI
Week 1
Week 5
Week 9

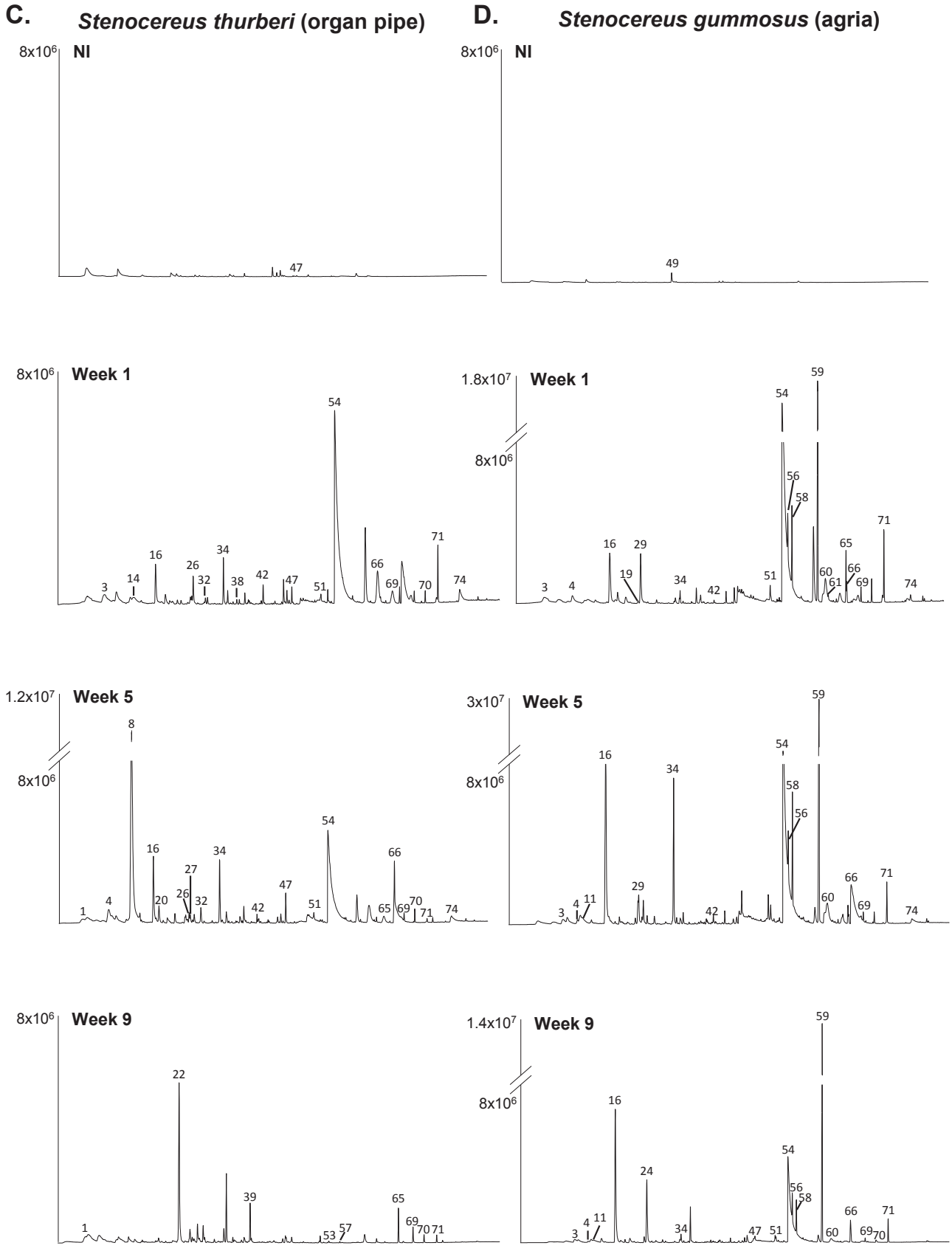
C.
Stenocereus thurberi (organ pipe)
D.
Stenocereus gummosus (agria)
8x10^6
NI
47
49
Week 1
Week 5
Week 9
1.8x10^7
3x10^7
1.2x10^7
1.4x10^7

Table S1. Analysis of variance for all experiments.

A. Barrel fermentation	Analysis	Source of Variation	d.f.	Mean squares	F ratio	p-value
Uninoculated vs. Fermentation week 1	Females	Line	1	48.400	9.68	0.0140
		Error	8	5.000		
	Males	Line	1	3.600	7.20	0.0270
		Error	8	0.500		
Fermentation week 1 vs. week 5	Females	Line	1	0.900	0.25	0.6320
		Error	8	3.650		
	Males	Line	1	0.900	0.95	0.3580
		Error	8	0.950		
Fermentation week 5 vs. week 9	Females	Line	1	0.400	0.40	0.5440
		Error	8	1.000		
	Males	Line	1	32.400	64.80	0.0001
		Error	8	0.500		
Fermentation week 1 vs. week 9	Females	Line	1	62.500	50.00	0.0001
		Error	8	1.250		
	Males	Line	1	40.000	19.51	0.0020
		Error	8	2.050		
B. Prickly pear fermentation	**Analysis**	**Source of Variation**	**d.f.**	**Mean squares**	**F ratio**	**p-value**
Uninoculated vs. Fermentation week 1	Females	Line	1	250.000	89.29	0.0001
		Error	8	2.800		
	Males	Line	1	78.400	28.51	0.0007
		Error	8	2.750		
Fermentation week 1 vs. week 5	Females	Line	1	10.000	2.32	0.1610
		Error	8	4.200		
	Males	Line	1	0.900	0.18	0.6820
		Error	8	5.000		
Fermentation week 5 vs. week 9	Females	Line	1	48.400	5.02	0.0500
		Error	8	9.650		
	Males	Line	1	52.900	12.03	0.0085
		Error	8	4.400		
Fermentation week 1 vs. week 9	Females	Line	1	184.900	63.76	0.0001
		Error	8	2.900		
	Males	Line	1	48.400	25.47	0.0010
		Error	8	1.900		
C. Organ pipe fermentation	**Analysis**	**Source of Variation**	**d.f.**	**Mean squares**	**F ratio**	**p-value**
Uninoculated vs. Fermentation week 5	Females	Line	1	78.400	39.20	0.0002

		Error	8	2.000		
	Males	Line	1	22.500	14.51	0.0052
		Error	8	1.550		
Fermentation week 1 vs. week 5	Females	Line	1	0.100	0.02	0.8882
		Error	8	4.700		
	Males	Line	1	3.600	0.45	0.5174
		Error	8	7.800		
Fermentation week 5 vs. week 9	Females	Line	1	10.000	1.04	0.3361
		Error	8	9.550		
	Males	Line	1	0.400	0.11	0.7404
		Error	8	3.400		
Fermentation week 1 vs. week 9	Females	Line	1	2.500	0.23	0.6395
		Error	8	10.550		
	Males	Line	1	0.100	0.02	0.8709
		Error	8	3.550		
D. Agria fermentation	**Analysis**	**Source of Variation**	**d.f.**	**Mean squares**	**F ratio**	**p-value**
Uninoculated vs. Fermentation week 1	Females	Line	1	32.400	6.00	0.0400
		Error	8	5.400		
	Males	Line	1	3.600	1.53	0.2509
		Error	8	2.350		
Fermentation week 1 vs. week 5	Females	Line	1	10.000	0.89	0.3724
		Error	8	11.200		
	Males	Line	1	16.900	1.50	0.2552
		Error	8	11.250		
Fermentation week 5 vs. week 9	Females	Line	1	19.600	3.26	0.1083
		Error	8	6.600		
	Males	Line	1	8.100	2.49	0.1531
		Error	8	3.250		
Fermentation week 1 vs. week 9	Females	Line	1	0.100	0.04	0.8417
		Error	8	2.350		
	Males	Line	1	16.900	1.55	0.2483
		Error	8	10.900		
E. Electrophysiological responses between line	**Analysis**	**Source of Variation**	**d.f.**	**Sum of squares**	**F ratio**	**p-value**
	Mojave	Line	1	0.220	0.23	0.6328
		Odor	10	36.520	3.75	0.0007
		Line X Odor	10	8.540	0.88	0.5599
	S Catalina	Line	1	1.287	1.91	0.1730
		Odor	10	61.564	9.12	0.0001
		Line X Odor	10	4.689	0.69	0.7253

F. Behavioral responses to the Synthetic mixture	Analysis	Source of Variation	d.f.	Mean squares	F ratio	p-value
10^{-2} Dilution	Females	Line	3	0.389	16.55	0.0001
		Error	40	0.023		
	Males	Line	3	0.031	2.73	0.0575
		Error	38	0.011		
10^{-3} Dilution	Females	Line	3	0.202	5.68	0.0020
		Error	40	0.035		
	Males	Line	3	0.011	0.86	0.4690
		Error	40	0.012		
10^{-4} Dilution	Females	Line	3	0.032	0.76	0.5240
		Error	40	0.042		
	Males	Line	3	0.005	0.26	0.8520
		Error	40	0.020		
G. Behavioral responses to the Single compounds	**Analysis**	**Source of Variation**	**d.f.**	**Mean squares**	**F ratio**	**p-value**
Acetone						
10^{-2} Dilution	Females	Line	3	0.167	2.67	0.0620
		Error	36	0.062		
	Males	Line	3	0.013	0.57	0.6380
		Error	36	0.023		
10^{-3} Dilution	Females	Line	3	0.017	0.28	0.8400
		Error	36	0.061		
	Males	Line	3	0.045	0.95	0.4250
		Error	36	0.047		
10^{-4} Dilution	Females	Line	3	0.057	0.92	0.4420
		Error	35	0.062		
	Males	Line	3	0.009	0.37	0.7760
		Error	36	0.024		
Isoamyl propionate						
10^{-2} Dilution	Females	Line	3	0.009	0.42	0.7380
		Error	40	0.021		
	Males	Line	3	0.012	0.82	0.4890
		Error	40	0.015		
10^{-3} Dilution	Females	Line	3	0.014	0.53	0.6619
		Error	31	0.026		
	Males	Line	3	0.012	0.81	0.4970
		Error	32	0.015		
10^{-4} Dilution	Females	Line	3	0.042	0.97	0.4170
		Error	40	0.043		
	Males	Line	3	0.026	1.71	0.1790
		Error	40	0.015		
Hexyl acetate						

10^{-2} Dilution	Females	Line	3	0.020	2.08	0.1202
		Error	36	0.010		
	Males	Line	3	0.008	0.90	0.4483
		Error	36	0.008		
10^{-3} Dilution	Females	Line	3	0.020	2.23	0.1012
		Error	36	0.010		
	Males	Line	3	0.001	0.37	0.7732
		Error	36	0.004		
10^{-4} Dilution	Females	Line	3	0.020	2.20	0.1051
		Error	36	0.010		
	Males	Line	3	0.010	2.09	0.1189
		Error	36	0.007		
1-hexanol						
10^{-2} Dilution	Females	Line	3	0.120	3.85	0.0173
		Error	36	0.030		
	Males	Line	3	0.020	3.22	0.0325
		Error	36	0.008		
10^{-3} Dilution	Females	Line	3	0.070	4.32	0.0106
		Error	36	0.010		
	Males	Line	3	0.026	3.88	0.0167
		Error	36	0.006		
10^{-4} Dilution	Females	Line	3	0.060	4.09	0.0135
		Error	36	0.010		
	Males	Line	3	0.010	1.53	0.2224
		Error	36	0.008		
2-nonanol						
10^{-2} Dilution	Females	Line	3	0.010	0.71	0.5498
		Error	36	0.020		
	Males	Line	3	0.004	0.69	0.5641
		Error	36	0.006		
10^{-3} Dilution	Females	Line	3	0.120	5.61	0.0029
		Error	36	0.020		
	Males	Line	3	0.010	1.40	0.2593
		Error	36	0.010		
10^{-4} Dilution	Females	Line	3	0.010	0.87	0.4666
		Error	35	0.010		
	Males	Line	3	0.002	0.48	0.7010
		Error	35	0.005		
Isopropyl benzoate						
10^{-2} Dilution	Females	Line	3	0.010	1.03	0.3928
		Error	36	0.010		
	Males	Line	3	0.008	0.91	0.4458
		Error	36	0.009		

10^{-3} Dilution	Females	Line	3	0.003	0.19	0.9000
		Error	36	0.010		
	Males	Line	3	0.001	0.09	0.9635
		Error	36	0.008		
10^{-4} Dilution	Females	Line	3	0.050	2.74	0.0576
		Error	36	0.010		
	Males	Line	3	0.005	0.61	0.6112
		Error	36	0.009		
Guaiacol						
10^{-2} Dilution	Females	Line	3	0.038	1.55	0.2170
		Error	36	0.024		
	Males	Line	3	0.032	1.03	0.3920
		Error	36	0.032		
10^{-3} Dilution	Females	Line	3	0.040	1.28	0.2970
		Error	32	0.031		
	Males	Line	3	0.006	0.43	0.7330
		Error	32	0.014		
10^{-4} Dilution	Females	Line	3	0.091	3.99	0.0152
		Error	35	0.022		
	Males	Line	3	0.009	0.42	0.7430
		Error	36	0.023		
Phenethyl propionate						
10^{-2} Dilution	Females	Line	3	0.030	1.87	0.1526
		Error	36	0.010		
	Males	Line	3	0.004	0.47	0.7118
		Error	36	0.008		
10^{-3} Dilution	Females	Line	3	0.034	1.25	0.3050
		Error	36	0.020		
	Males	Line	3	0.007	0.75	0.5310
		Error	36	0.009		
10^{-4} Dilution	Females	Line	3	0.030	1.45	0.2446
		Error	36	0.020		
	Males	Line	3	0.010	1.14	0.3464
		Error	36	0.010		
Phenol						
10^{-2} Dilution	Females	Line	3	0.070	2.86	0.0506
		Error	36	0.020		
	Males	Line	3	0.010	1.48	0.2352
		Error	36	0.010		
10^{-3} Dilution	Females	Line	3	0.010	1.34	0.2767
		Error	36	0.010		
	Males	Line	3	0.009	0.98	0.4100
		Error	36	0.009		

10^{-4} Dilution	Females	Line	3	0.100	5.49	0.0033
		Error	36	0.010		
	Males	Line	3	0.007	1.22	0.3190
		Error	36	0.006		
4-ethylguaiacol						
10^{-2} Dilution	Females	Line	3	0.255	8.47	0.0002
		Error	35	0.030		
	Males	Line	3	0.012	0.88	0.4590
		Error	36	0.013		
10^{-3} Dilution	Females	Line	3	0.061	2.48	0.0775
		Error	35	0.025		
	Males	Line	3	0.120	3.01	0.0429
		Error	35	0.039		
10^{-4} Dilution	Females	Line	3	0.042	1.04	0.3870
		Error	35	0.041		
	Males	Line	3	0.041	2.09	0.1180
		Error	36	0.019		
4-ethyl phenol						
10^{-2} Dilution	Females	Line	3	0.003	0.10	0.9623
		Error	36	0.030		
	Males	Line	3	0.010	2.40	0.0837
		Error	36	0.010		
10^{-3} Dilution	Females	Line	3	0.010	0.36	0.7776
		Error	36	0.040		
	Males	Line	3	0.003	0.29	0.8307
		Error	36	0.010		
10^{-4} Dilution	Females	Line	3	0.010	0.30	0.8228
		Error	36	0.030		
	Males	Line	3	0.005	0.22	0.8835
		Error	36	0.020		
4-vinyl guaiacol						
10^{-2} Dilution	Females	Line	3	0.020	1.64	0.1962
		Error	36	0.010		
	Males	Line	3	0.009	1.65	0.1942
		Error	36	0.005		
10^{-3} Dilution	Females	Line	3	0.030	1.76	0.1730
		Error	36	0.010		
	Males	Line	3	0.000	0.06	0.9806
		Error	36	0.006		
10^{-4} Dilution	Females	Line	3	0.010	0.75	0.5314
		Error	36	0.010		
	Males	Line	3	0.004	1.07	0.3728
		Error	36	0.004		

Phenethyl acetate						
10^{-2} Dilution	Females	Line	3	0.087	3.23	0.0340
		Error	35	0.027		
	Males	Line	3	0.009	0.75	0.5270
		Error	35	0.012		
10^{-3} Dilution	Females	Line	3	0.357	10.19	0.0001
		Error	28	0.035		
	Males	Line	3	0.035	3.16	0.0400
		Error	28	0.011		
10^{-4} Dilution	Females	Line	3	0.063	2.46	0.0790
		Error	34	0.025		
	Males	Line	3	0.023	1.12	0.3550
		Error	35	0.020		

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Class	Uninoculated Barrel cactus	Week one Barrel cactus rot	Week two Barrel cactus rot	Week three Barrel cactus rot	Week four Barrel cactus rot
1	Acetone	ketone	0 ± 0	0 ± 0	4.83 ± 1.29	9.48 ± 13.40	8.70 ± 12.25
2	2-butanone	ketone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	2-propanol	alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Propyl acetate	ester	0 ± 0	0 ± 0	14.71 ± 0.37	12.21 ± 17.26	0 ± 0
5	Isopropyl acetate	ester	0 ± 0	0 ± 0	0 ± 0	10.29 ± 14.56	0 ± 0
6	Ethyl propionate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	ester	0 ± 0	0 ± 0	0 ± 0	4.75 ± 6.71	10.44 ± 14.76
8	Propyl propionate	ester	0 ± 0	0 ± 0	7.02 ± 1.03	2.37 ± 3.35	0 ± 0
9	6-methyl-2 Hepatanone	ketone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	other	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	Isopropyl isopentanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
17	Isopentyl acetate	ester	0 ± 0	0 ± 0	11.30 ± 7.47	0.37 ± 0.52	13.77 ± 19.30
18	1-undecene	other	0 ± 0	1.85 ± 1.28	0 ± 0	0.09 ± 0.13	0 ± 0
19	1-dodecene	other	1.36 ± 1.86	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	ester	0 ± 0	0 ± 0	1.78 ± 2.51	1.82 ± 2.57	21.62 ± 18.77
23	2-heptanone	ketone	0.23 ± 0.02	0 ± 0	0 ± 0	0.39 ± 0.55	0 ± 0
24	Butyl butyrate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
25	Isopentyl alcohol	alcohol	0.14 ± 0.19	0 ± 0	0.90 ± 0.18	0.96 ± 0.05	0 ± 0
26	Hexanoic acid ethyl ester	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
27	Hexanoic acid 1-methylethyl ester	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
28	Isopropyl tiglate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Class	Uninoculated Barrel cactus	Week one Barrel cactus rot	Week two Barrel cactus rot	Week three Barrel cactus rot	Week four Barrel cactus rot
29	Isopentyl butyrate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	ester	0 ± 0	0.12 ± 0.16	2.92 ± 1.39	1.40 ± 1.99	0 ± 0
31	3-octanone	ketone	0.35 ± 0.49	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	ester	0 ± 0	1.42 ± 0.08	1.54 ± 0.82	0.56 ± 0.79	0.33 ± 0.46
33	Acetoin	ketone	0.58 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0
34	n-propyl hexanoate	ester	0 ± 0	0.10 ± 0.14	0 ± 0	0 ± 0	0.60 ± 0.84
35	Isobutyl tiglate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	alcohol	0 ± 0	0.43 ± 0.04	0 ± 0	0.17 ± 0.24	0 ± 0
39	2-nonanone	ketone	1.46 ± 1.93	1.50 ± 1.18	2.94 ± 3.02	2.55 ± 2.78	1.39 ± 1.96
40	Durenol	aromatic	0 ± 0	2.87 ± 0.40	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
43	Isopentyl hexanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	aromatic	0 ± 0	0.77 ± 0.39	0 ± 0	1.01 ± 1.43	1.21 ± 0.85
46	2-nonanol	alcohol	0 ± 0	2.91 ± 1.06	1.73 ± 0.03	1.81 ± 1.72	0 ± 0
47	Propyl octanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
48	Linalool	other	0 ± 0	0 ± 0	0.08 ± 0.12	0.03 ± 0.04	0 ± 0
49	Benzaldehyde	aromatic	0.35 ± 0.50	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
52	n-propyl, 3-mercapto-propanoate	ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	aromatic	0 ± 0	0.63 ± 0.30	2.60 ± 0.08	0.41 ± 0.37	1.10 ± 1.56
54	Butyric acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
55	Eugenol	aromatic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	aromatic	0 ± 0	0 ± 0	1.34 ± 0.49	0.98 ± 1.38	1.87 ± 1.83
57	Acetophenone	aromatic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
58	Ethyl benzoate	aromatic	0 ± 0	4.07 ± 2.18	4.59 ± 0.93	7.37 ± 6.19	0.79 ± 1.11
59	Propyl benzoate	aromatic	0 ± 0	6.41 ± 2.28	2.23 ± 0.94	1.21 ± 0.26	0.37 ± 0.53
60	Pentanoic acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
61	Butyl benzoate	aromatic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
62	Methyl salicylate	aromatic	0 ± 0	0.31 ± 0.23	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	ketone	0 ± 0	0.22 ± 0.09	0.48 ± 0.04	0.42 ± 0.15	0.50 ± 0.19
64	2-phenethyl acetate	aromatic	0 ± 0	0.14 ± 0.01	2.44 ± 0.06	1.80 ± 2.14	4.91 ± 3.94
65	2-methoxy phenol	aromatic	0 ± 0	6.03 ± 0.41	5.96 ± 3.69	4.34 ± 1.00	3.38 ± 1.90
66	Hexanoic acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Class	Uninoculated Barrel cactus	Week one Barrel cactus rot	Week two Barrel cactus rot	Week three Barrel cactus rot	Week four Barrel cactus rot
67	Phenethyl propionate	aromatic	0 ± 0	0 ± 0	0.85 ± 0.07	0.68 ± 0.96	8.40 ± 2.19
68	Isopentyl benzoate	aromatic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
69	Phenethyl alcohol	aromatic	0 ± 0	0.11 ± 0.01	0.11 ± 0.16	0.23 ± 0.16	0.31 ± 0.26
70	Creosol	aromatic	0 ± 0	0.24 ± 0.11	0.21 ± 0.12	0.25 ± 0.19	0.16 ± 0.14
71	Phenol	aromatic	0 ± 0	2.45 ± 0.08	2.19 ± 1.10	2.00 ± 1.58	1.61 ± 0.79
72	4-ethylguaiacol	aromatic	0 ± 0	2.97 ± 0.91	1.80 ± 1.66	1.75 ± 1.22	3.91 ± 2.24
73	4-methyl phenol	aromatic	0 ± 0	0.10 ± 0.00	0.13 ± 0.05	0.11 ± 0.06	0.13 ± 0.06
74	Octanoic acid	acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	aromatic	0 ± 0	0.22 ± 0.16	0.03 ± 0.04	0.06 ± 0.09	0.18 ± 0.07
76	2-methoxy-4-propyl phenol	aromatic	0 ± 0	0.04 ± 0.06	0.05 ± 0.07	0 ± 0	0.01 ± 0.02
77	4-vinylguaiacol	aromatic	0 ± 0	0.18 ± 0.02	0.10 ± 0.03	0 ± 0	0.08 ± 0.11

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Barrel cactus rot	Week six Barrel cactus rot	Week seven Barrel cactus rot	Week eight Barrel cactus rot	Week nine Barrel cactus rot
1	Acetone	6.16 ± 8.71	4.83 ± 1.91	15.72 ± 1.50	9.97 ± 4.18	14.51 ± 20.52
2	2-butanone	4.25 ± 0.51	0 ± 0	1.88 ± 2.66	0 ± 0	5.07 ± 7.17
3	2-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Propyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	Isopropyl acetate	0 ± 0	5.82 ± 3.06	0 ± 0	8.68 ± 12.27	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	7.92 ± 1.43	40.11 ± 51.59	17.73 ± 10.52	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.38 ± 0.54
9	6-methyl-2 Hepatanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	0.09 ± 0.12	0.76 ± 1.07	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
17	Isopentyl acetate	2.04 ± 2.53	9.27 ± 0.78	12.40 ± 14.44	15.64 ± 8.96	0.34 ± 0.48
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	2.60 ± 0.02	17.28 ± 11.61	41.99 ± 53.67	13.99 ± 17.87	0.32 ± 0.08
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	Butyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
25	Isopentyl alcohol	13.43 ± 14.32	0.56 ± 0.80	1.56 ± 2.21	1.56 ± 0.05	0.91 ± 0.65
26	Hexanoic acid ethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
27	Hexanoic acid 1-methylethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Barrel cactus rot	Week six Barrel cactus rot	Week seven Barrel cactus rot	Week eight Barrel cactus rot	Week nine Barrel cactus rot
29	Isopentyl butyrate	0.18 ± 0.25	0 ± 0	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
31	3-octanone	0.12 ± 0.17	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0 ± 0	0.13 ± 0.19	0 ± 0	0.27 ± 0.38
33	Acetoin	0.83 ± 0.59	0 ± 0	0 ± 0	0 ± 0	1.77 ± 1.51
34	n-propyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
39	2-nonanone	1.75 ± 1.31	0.54 ± 0.77	1.43 ± 2.03	1.43 ± 1.12	0.12 ± 0.17
40	Durenol	0.68 ± 0.96	0.37 ± 0.52	0.53 ± 0.74	0 ± 0	3.07 ± 2.40
41	Butyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	1.40 ± 1.98	1.42 ± 1.54	1.73 ± 1.62	0.72 ± 1.01	2.71 ± 0.91
46	2-nonanol	0.41 ± 0.01	0 ± 0	0.20 ± 0.14	0.10 ± 0.02	0 ± 0
47	Propyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
48	Linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.06 ± 0.08
49	Benzaldehyde	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.05 ± 0.06
50	Acetic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	5.02 ± 3.94	1.70 ± 1.61	1.03 ± 1.45	1.30 ± 0.56	0 ± 0
54	Butyric acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
55	Eugenol	0.20 ± 0.28	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	1.22 ± 1.73	1.36 ± 0.68	0.95 ± 0.62	0.78 ± 0.63	0 ± 0
57	Acetophenone	0.16 ± 0.23	0 ± 0	0 ± 0	0.11 ± 0.16	0.11 ± 0.16
58	Ethyl benzoate	0.32 ± 0.46	0 ± 0	0.18 ± 0.25	0.08 ± 0.11	0 ± 0
59	Propyl benzoate	0 ± 0	0 ± 0	0.25 ± 0.36	0.24 ± 0.34	0 ± 0
60	Pentanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
61	Butyl benzoate	0.37 ± 0.52	0.27 ± 0.38	0.40 ± 0.56	0 ± 0	0 ± 0
62	Methyl salicylate	0.10 ± 0.05	0 ± 0	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	0.29 ± 0.04	0.22 ± 0.32	0 ± 0	0.33 ± 0.09	0.11 ± 0.15
64	2-phenethyl acetate	1.71 ± 2.05	3.37 ± 3.44	3.28 ± 3.50	3.44 ± 0.08	0.65 ± 0.14
65	2-methoxy phenol	5.96 ± 1.31	2.97 ± 1.09	4.20 ± 1.64	2.68 ± 1.10	12.14 ± 2.87
66	Hexanoic acid	0.14 ± 0.20	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Barrel cactus rot	Week six Barrel cactus rot	Week seven Barrel cactus rot	Week eight Barrel cactus rot	Week nine Barrel cactus rot
67	Phenethyl propionate	0.79 ± 0.58	7.38 ± 7.07	9.97 ± 11.03	7.15 ± 2.21	0.23 ± 0.32
68	Isopentyl benzoate	1.86 ± 2.15	0 ± 0	0.12 ± 0.16	0.09 ± 0.13	0 ± 0
69	Phenethyl alcohol	3.79 ± 1.48	0.22 ± 0.31	0.18 ± 0.26	0.32 ± 0.08	1.19 ± 0.24
70	Creosol	0.46 ± 0.37	0.42 ± 0.39	0.82 ± 0.91	1.38 ± 1.69	0.25 ± 0.08
71	Phenol	1.64 ± 0.57	1.78 ± 0.16	2.45 ± 0.42	1.91 ± 0.08	2.29 ± 0.74
72	4-ethylguaiacol	63.07 ± 35.29	2.98 ± 2.09	10.05 ± 7.92	2.43 ± 1.14	115.55 ± 105.80
73	4-methyl phenol	0.09 ± 0.01	0.21 ± 0.11	0.16 ± 0.08	0.17 ± 0.04	0.23 ± 0.01
74	Octanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	0.15 ± 0.04	0.13 ± 0.19	0.03 ± 0.05	0 ± 0	0.65 ± 0.60
76	2-methoxy-4-propyl phenol	0.16 ± 0.23	0 ± 0	0.14 ± 0.04	0.11 ± 0.02	0.22 ± 0.31
77	4-vinylguaiacol	0.13 ± 0.02	0.26 ± 0.13	0.14 ± 0.04	0.07 ± 0.10	0.33 ± 0.30

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Prickly pear cactus	Week one Prickly pear cactus rot	Week two Prickly pear cactus rot	Week three Prickly pear cactus rot	Week four Prickly pear cactus rot
1	Acetone	0 ± 0	0.57 ± 0.81	2.44 ± 2.53	3.98 ± 1.36	7.72 ± 8.65
2	2-butanone	0 ± 0	0 ± 0	11.14 ± 3.54	18.83 ± 23.21	4.36 ± 0.45
3	2-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Propyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	3.38E-07 ± 0.00	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	19.42 ± 27.46	0 ± 0	0 ± 0
9	6-methyl-2 Hepatanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	6.04 ± 0.09	0 ± 0	0 ± 0	0 ± 0
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	0.19 ± 0.27	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
17	Isopentyl acetate	0 ± 0	0 ± 0	9.30 ± 13.16	0.69 ± 0.97	0 ± 0
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0 ± 0	0 ± 0	14.43 ± 20.41	1.31 ± 1.85	0 ± 0
23	2-heptanone	1.07 ± 0.06	0 ± 0	0.11 ± 0.15	0 ± 0	0 ± 0
24	Butyl butyrate	5.82 ± 0.16	12.07 ± 2.26	0 ± 0	0 ± 0	0 ± 0
25	Isopentyl alcohol	0 ± 0	0.82 ± 1.17	1.36 ± 1.05	2.28 ± 3.23	0.17 ± 0.24
26	Hexanoic acid ethyl ester	0 ± 0	2.00 ± 0.66	0 ± 0	0 ± 0	0 ± 0
27	Hexanoic acid 1-methylethyl ester	0 ± 0	0.65 ± 0.93	0 ± 0	0 ± 0	1.50 ± 0.84
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Prickly pear cactus	Week one Prickly pear cactus rot	Week two Prickly pear cactus rot	Week three Prickly pear cactus rot	Week four Prickly pear cactus rot
29	Isopentyl butyrate	0 ± 0	7.16 ± 0.27	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
31	3-octanone	1.23 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0.65 ± 0.92	0.54 ± 0.76	0 ± 0	0 ± 0
33	Acetoin	3.29 ± 1.42	0.33 ± 0.47	0.62 ± 0.88	0.10 ± 0.15	0 ± 0
34	n-propyl hexanoate	0 ± 0	0.68 ± 0.41	0 ± 0	0 ± 0	0 ± 0
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	1.01 ± 0.37	0.14 ± 0.20	0 ± 0	0 ± 0	0 ± 0
39	2-nonanone	9.13 ± 0.86	0.23 ± 0.16	0 ± 0	0.37 ± 0.52	0 ± 0
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	1.74 ± 1.77	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
43	Isopentyl hexanoate	0 ± 0	0.69 ± 0.62	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	1.21 ± 0.11	0 ± 0	0 ± 0	0.07 ± 0.09	0 ± 0
47	Propyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
48	Linalool	1.22 ± 0.50	5.72 ± 0.24	0.98 ± 0.55	1.49 ± 0.35	8.34 ± 6.09
49	Benzaldehyde	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	0 ± 0	0.17 ± 0.24	0.17 ± 0.24	0.21 ± 0.30	0 ± 0
54	Butyric acid	0 ± 0	50.13 ± 20.58	0 ± 0	0 ± 0	0 ± 0
55	Eugenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	0 ± 0	5.46 ± 3.06	0.08 ± 0.11	0.45 ± 0.38	0.70 ± 0.23
57	Acetophenone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.04 ± 0.05
58	Ethyl benzoate	0 ± 0	2.62 ± 1.70	1.30 ± 1.84	0.20 ± 0.09	0 ± 0
59	Propyl benzoate	0 ± 0	0.14 ± 0.04	0.68 ± 0.96	0.14 ± 0.02	0 ± 0
60	Pentanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
61	Butyl benzoate	0 ± 0	0.03 ± 0.04	0 ± 0	0 ± 0	0 ± 0
62	Methyl salicylate	0.13 ± 0.03	3.07 ± 0.41	0.37 ± 0.53	0.27 ± 0.16	0.21 ± 0.29
63	2-tridecanone	0.65 ± 0.16	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	2-phenethyl acetate	0 ± 0	1.03 ± 1.24	0 ± 0	2.75 ± 0.79	0.38 ± 0.54
65	2-methoxy phenol	0.13 ± 0.11	0 ± 0	0.18 ± 0.25	0 ± 0	0 ± 0
66	Hexanoic acid	0 ± 0	7.82 ± 4.93	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Prickly pear cactus	Week one Prickly pear cactus rot	Week two Prickly pear cactus rot	Week three Prickly pear cactus rot	Week four Prickly pear cactus rot
67	Phenethyl propionate	0 ± 0	0.51 ± 0.71	9.11 ± 12.88	2.26 ± 0.13	0 ± 0
68	Isopentyl benzoate	0 ± 0	0.10 ± 0.14	0.08 ± 0.12	0.43 ± 0.60	0 ± 0
69	Phenethyl alcohol	0.31 ± 0.05	0.59 ± 0.36	0.21 ± 0.08	5.82 ± 1.22	0.37 ± 0.35
70	Creosol	0 ± 0	0 ± 0	0.02 ± 0.03	0.03 ± 0.04	0 ± 0
71	Phenol	0.42 ± 0.59	0.68 ± 0.21	0.42 ± 0.26	0.57 ± 0.17	0.34 ± 0.23
72	4-ethylguaiacol	0.03 ± 0.04	0.08 ± 0.00	0.69 ± 0.98	0 ± 0	0 ± 0
73	4-methyl phenol	0 ± 0	13.88 ± 19.19	0.93 ± 1.31	3.48 ± 4.80	23.73 ± 28.71
74	Octanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	0 ± 0	1.06 ± 0.25	0 ± 0	0.44 ± 0.21	0.90 ± 1.06
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Prickly pear cactus rot	Week six Prickly pear cactus rot	Week seven Prickly pear cactus rot	Week eight Prickly pear cactus rot	Week nine Prickly pear cactus rot
1	Acetone	12.47 ± 15.84	12.50 ± 4.96	9.45 ± 1.33	2.26 ± 3.19	19.43 ± 27.48
2	2-butanone	11.48 ± 12.33	4.74 ± 6.70	14.77 ± 18.43	0 ± 0	4.44 ± 6.28
3	2-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Propyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
9	6-methyl-2 Hepatanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	Isopropyl isopentanoate	0.76 ± 1.07	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	2.58 ± 3.65	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	37.58 ± 53.15
16	Propyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
17	Isopentyl acetate	0.42 ± 0.59	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	0.29 ± 0.41	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0.04 ± 0.05	0 ± 0	0 ± 0	0 ± 0	3.46 ± 4.44
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	Butyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
25	Isopentyl alcohol	0.78 ± 1.11	0.14 ± 0.19	0.36 ± 0.20	0 ± 0	10.44 ± 0.19
26	Hexanoic acid ethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
27	Hexanoic acid 1-methylethyl ester	1.56 ± 2.20	0 ± 0	0 ± 0	0 ± 0	0 ± 0
28	Isopropyl tiglate	0.40 ± 0.13	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Prickly pear cactus rot	Week six Prickly pear cactus rot	Week seven Prickly pear cactus rot	Week eight Prickly pear cactus rot	Week nine Prickly pear cactus rot
29	Isopentyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.59 ± 0.84
31	3-octanone	0.10 ± 0.15	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.14 ± 0.20
33	Acetoin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.27 ± 0.38
34	n-propyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
35	Isobutyl tiglate	0.52 ± 0.74	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	0.12 ± 0.16	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
39	2-nonanone	0.14 ± 0.05	0.07 ± 0.09	0.07 ± 0.10	0 ± 0	0.73 ± 0.65
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.09 ± 0.12
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.20 ± 0.28
47	Propyl octanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
48	Linalool	0.55 ± 0.15	7.33 ± 0.93	3.81 ± 4.99	9.01 ± 6.54	0.27 ± 0.02
49	Benzaldehyde	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.66 ± 0.93
53	Methyl benzoate	0.16 ± 0.07	0 ± 0	0.51 ± 0.73	0 ± 0	0.68 ± 0.96
54	Butyric acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
55	Eugenol	0.24 ± 0.34	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	0.39 ± 0.55	0.08 ± 0.12	1.20 ± 0.36	0.12 ± 0.17	0.65 ± 0.91
57	Acetophenone	0.04 ± 0.06	0.03 ± 0.04	0.05 ± 0.07	0 ± 0	0 ± 0
58	Ethyl benzoate	0.70 ± 0.81	0 ± 0	0 ± 0	0.13 ± 0.18	0.25 ± 0.04
59	Propyl benzoate	0 ± 0	0 ± 0	0 ± 0	0.04 ± 0.06	0 ± 0
60	Pentanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
61	Butyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.10 ± 0.15
62	Methyl salicylate	1.07 ± 0.66	0.06 ± 0.08	0.04 ± 0.05	0 ± 0	2.66 ± 2.32
63	2-tridecanone	0 ± 0	0 ± 0	0.07 ± 0.09	0 ± 0	0.06 ± 0.09
64	2-phenethyl acetate	1.38 ± 1.37	0.07 ± 0.10	0.19 ± 0.27	0 ± 0	18.68 ± 26.11
65	2-methoxy phenol	0.22 ± 0.31	0 ± 0	0.36 ± 0.51	0 ± 0	0.13 ± 0.03
66	Hexanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.12 ± 0.18

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Prickly pear cactus rot	Week six Prickly pear cactus rot	Week seven Prickly pear cactus rot	Week eight Prickly pear cactus rot	Week nine Prickly pear cactus rot
67	Phenethyl propionate	0.32 ± 0.25	0 ± 0	0 ± 0	0 ± 0	1.71 ± 2.42
68	Isopentyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.26 ± 0.36
69	Phenethyl alcohol	2.89 ± 0.43	1.48 ± 1.68	1.78 ± 0.83	1.35 ± 0.86	1.60 ± 1.80
70	Creosol	0.10 ± 0.14	0 ± 0	0.01 ± 0.02	0 ± 0	0 ± 0
71	Phenol	0.76 ± 0.63	0.30 ± 0.12	0.34 ± 0.02	0.20 ± 0.07	0.59 ± 0.45
72	4-ethylguaiacol	0.46 ± 0.65	0.01 ± 0.01	0.18 ± 0.26	0 ± 0	1.49 ± 1.81
73	4-methyl phenol	1.88 ± 0.99	195.72 ± 65.71	86.73 ± 111.44	105.49 ± 34.48	0.07 ± 0.03
74	Octanoic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	0.38 ± 0.13	1.09 ± 0.22	2.06 ± 0.51	1.92 ± 0.52	0.75 ± 0.15
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.11 ± 0.16

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Organ Pipe cactus	Week one Organ Pipe cactus rot	Week two Organ Pipe cactus rot	Week three Organ Pipe cactus rot	Week four Organ Pipe cactus rot
1	Acetone	0 ± 0	0 ± 0	0 ± 0	2.33 ± 4.04	1.55 ± 1.35
2	2-butanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	2-propanol	0 ± 0	3.84 ± 1.84	2.5 ± 1.12	0 ± 0	0 ± 0
4	Propyl acetate	0 ± 0	0 ± 0	7.21 ± 2.78	7.05 ± 3.3	7.57 ± 2.11
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	34.76 ± 8.11	48.67 ± 7.44	56.89 ± 8.61
9	6-methyl-2 Hepatanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	0.33 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	2.02 ± 3.51	0.16 ± 0.27	0.12 ± 0.22	0 ± 0
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	2.27 ± 3.93	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	0 ± 0	3.1 ± 0.45	4.76 ± 1.85	4.21 ± 2.41	5.34 ± 1.75
17	Isopentyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.01
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0.89 ± 0.36	1.17 ± 0.56	1.65 ± 0.27
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0 ± 0	0 ± 0	0.3 ± 0.53	0.15 ± 0.27	0.86 ± 0.51
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	Butyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.22 ± 0.22
25	Isopentyl alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.08
26	Hexanoic acid ethyl ester	0 ± 0	0.79 ± 0.37	0.53 ± 0.49	0.13 ± 0.23	0.63 ± 0.38
27	Hexanoic acid 1-methylethyl ester	0 ± 0	0.1 ± 0.17	0.17 ± 0.3	0 ± 0	0.21 ± 0.37
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Organ Pipe cactus	Week one Organ Pipe cactus rot	Week two Organ Pipe cactus rot	Week three Organ Pipe cactus rot	Week four Organ Pipe cactus rot
29	Isopentyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
31	3-octanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0.27 ± 0.07	0.62 ± 0.16	0.52 ± 0.28	0.64 ± 0.61
33	Acetoin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
34	n-propyl hexanoate	0 ± 0	1.35 ± 0.65	2.84 ± 0.71	3.09 ± 0.29	3.09 ± 0.69
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0.53 ± 0.55	0.61 ± 0.54	0.71 ± 0.76
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0.17 ± 0.02	0 ± 0	0 ± 0	0 ± 0
39	2-nonanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	0 ± 0	0.02 ± 0.04	0.03 ± 0.05	0 ± 0
42	Ethyl octanoate	0 ± 0	0.27 ± 0.24	0.44 ± 0.22	0.55 ± 0.06	0.26 ± 0.15
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0.02 ± 0.04	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
47	Propyl octanoate	0 ± 0	0.36 ± 0.23	1.57 ± 1.32	1.31 ± 0.23	1.09 ± 0.43
48	Linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
49	Benzaldehyde	0.1 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	0 ± 0	0.77 ± 0.73	2.84 ± 1.09	2.84 ± 1.55	4.14 ± 1.33
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
54	Butyric acid	0 ± 0	26.61 ± 5.17	33.87 ± 15.51	26.44 ± 15.73	26.37 ± 9.04
55	Eugenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
57	Acetophenone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
58	Ethyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
59	Propyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.04
60	Pentanoic acid	0 ± 0	0 ± 0	0 ± 0	0.87 ± 1.5	1.7 ± 2.94
61	Butyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
62	Methyl salicylate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	2-phenethyl acetate	0 ± 0	0 ± 0	0.31 ± 0.31	0.14 ± 0.15	0.3 ± 0.16
65	2-methoxy phenol	0 ± 0	0.09 ± 0.13	0.09 ± 0.11	0.12 ± 0.14	0.11 ± 0.14
66	Hexanoic acid	0 ± 0	4.37 ± 2.25	5.1 ± 2.12	6.77 ± 2.61	6.67 ± 0.47

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Organ Pipe cactus	Week one Organ Pipe cactus rot	Week two Organ Pipe cactus rot	Week three Organ Pipe cactus rot	Week four Organ Pipe cactus rot
67	Phenethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
68	Isopentyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
69	Phenethyl alcohol	0 ± 0	0.22 ± 0.05	0.05 ± 0.08	0 ± 0	0.16 ± 0.15
70	Creosol	0 ± 0	0.19 ± 0.03	0.21 ± 0.03	0.25 ± 0.04	0.16 ± 0.13
71	Phenol	0 ± 0	0.41 ± 0.64	0.46 ± 0.57	0.73 ± 1.02	0.81 ± 1.18
72	4-ethylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.04 ± 0.04
73	4-methyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
74	Octanoic acid	0 ± 0	0.89 ± 0.54	1.26 ± 0.13	1.43 ± 0.63	1.35 ± 0.19
75	4-ethyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Organ Pipe cactus rot	Week six Organ Pipe cactus rot	Week seven Organ Pipe cactus rot	Week eight Organ Pipe cactus rot	Week nine Organ Pipe cactus rot
1	Acetone	0.89 ± 1.54	2.93 ± 0.68	1.57 ± 1.47	2.72 ± 2.1	5.36 ± 1.05
2	2-butanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	2-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Propyl acetate	3.55 ± 4.88	1.75 ± 3.03	0.53 ± 0.49	0 ± 0	0 ± 0
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	32.36 ± 16.06	27 ± 9.67	3.32 ± 3.12	0.15 ± 0.26	0 ± 0
9	6-methyl-2 Hepatanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	Hexanal	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	Isobutanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	3.53 ± 1.73	2.91 ± 1.6	1.07 ± 0.93	0.06 ± 0.1	0 ± 0
17	Isopentyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.15 ± 0.27
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	1.14 ± 0.75	1.07 ± 1.21	0.23 ± 0.4	1.57 ± 2.17	1.36 ± 1.2
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0.25 ± 0.43	0.85 ± 1.48	0 ± 0	0 ± 0	5.09 ± 8.81
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.29 ± 0.51
24	Butyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
25	Isopentyl alcohol	0 ± 0	0 ± 0	0 ± 0	0.48 ± 0.83	0.35 ± 0.6
26	Hexanoic acid ethyl ester	0.37 ± 0.19	0.35 ± 0.36	0.17 ± 0.29	0 ± 0	0 ± 0
27	Hexanoic acid 1-methylethyl ester	0.28 ± 0.16	0.22 ± 0.3	0.15 ± 0.26	0 ± 0	0 ± 0
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.13 ± 0.22

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Organ Pipe cactus rot	Week six Organ Pipe cactus rot	Week seven Organ Pipe cactus rot	Week eight Organ Pipe cactus rot	Week nine Organ Pipe cactus rot
29	Isopentyl butyrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0.03 ± 0.05	0 ± 0	0 ± 0
31	3-octanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0.58 ± 0.88	1.91 ± 3.3	0.24 ± 0.41	0 ± 0	0 ± 0
33	Acetoin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
34	n-propyl hexanoate	2.24 ± 0.82	1.1 ± 0.98	0 ± 0	0 ± 0	0 ± 0
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	1.13 ± 1.95	1.25 ± 2.16	0.07 ± 0.11	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
39	2-nonanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.72 ± 1.25
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	0.16 ± 0.19	0.04 ± 0.08	0 ± 0	0 ± 0	0 ± 0
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
47	Propyl octanoate	0.76 ± 0.41	0.13 ± 0.11	0 ± 0	0 ± 0	0 ± 0
48	Linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
49	Benzaldehyde	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
51	Propionic acid	3.04 ± 1.57	1.17 ± 0.4	0.53 ± 0.49	0.59 ± 0.93	0 ± 0
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.04 ± 0.06
54	Butyric acid	14.3 ± 7.31	3.69 ± 1.17	1.36 ± 0.57	1.58 ± 2.05	0.56 ± 0.97
55	Eugenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
57	Acetophenone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.04 ± 0.04
58	Ethyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
59	Propyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
60	Pentanoic acid	0.65 ± 1.12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
61	Butyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
62	Methyl salicylate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	2-phenethyl acetate	0.07 ± 0.12	0.23 ± 0.4	0.2 ± 0.35	0 ± 0	0 ± 0
65	2-methoxy phenol	0.24 ± 0.37	0.2 ± 0.35	1.35 ± 1.69	0.79 ± 0.71	1.57 ± 1.79
66	Hexanoic acid	4.07 ± 0.71	1.09 ± 0.16	0.11 ± 0.2	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Organ Pipe cactus rot	Week six Organ Pipe cactus rot	Week seven Organ Pipe cactus rot	Week eight Organ Pipe cactus rot	Week nine Organ Pipe cactus rot
67	Phenethyl propionate	0 ± 0	0.49 ± 0.27	0 ± 0	0 ± 0	0 ± 0
68	Isopentyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
69	Phenethyl alcohol	0.22 ± 0.19	0.18 ± 0.15	0.24 ± 0.07	0.21 ± 0.19	0.68 ± 0.27
70	Creosol	0.16 ± 0.14	0.13 ± 0.11	0.11 ± 0.11	0.1 ± 0.08	0.25 ± 0.23
71	Phenol	1.11 ± 1.81	1.09 ± 1.72	2.55 ± 1.34	2.27 ± 1.38	1.56 ± 1.11
72	4-ethylguaiacol	0.05 ± 0.04	0.02 ± 0.04	0.03 ± 0.03	0.03 ± 0.02	0.07 ± 0.06
73	4-methyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
74	Octanoic acid	0.71 ± 0.17	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Agria cactus	Week one Agria cactus rot	Week two Agria cactus rot	Week three Agria cactus rot	Week four Agria cactus rot
1	Acetone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2	2-butanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	2-propanol	0 ± 0	2.78 ± 1.06	1.57 ± 1.62	3.62 ± 0.61	2.21 ± 2.14
4	Propyl acetate	0 ± 0	0.37 ± 0.65	1.46 ± 1.57	2.83 ± 0.89	2.4 ± 1.9
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
9	6-methyl-2 Hepatanone	1.33 ± 0.18	0.04 ± 0.03	0.38 ± 0.44	0.8 ± 0.55	1.01 ± 1.01
10	Hexanal	0.78 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	0 ± 0	0.35 ± 0.6	0.94 ± 0.84	1.28 ± 1.28	1 ± 0.8
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	0 ± 0	2.8 ± 4.85	1.24 ± 2.15	4.74 ± 2.29	5.05 ± 3.62
15	Isobutanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	0 ± 0	4.73 ± 4.27	6.56 ± 6.89	20.16 ± 2.27	10.55 ± 7.53
17	Isopentyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0.11 ± 0.19	0 ± 0	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	Butyl butyrate	0 ± 0	2.93 ± 2.08	2.2 ± 3.81	4.92 ± 3.51	2.99 ± 4.61
25	Isopentyl alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
26	Hexanoic acid ethyl ester	0 ± 0	0 ± 0	0.11 ± 0.19	0 ± 0	0.05 ± 0.09
27	Hexanoic acid 1-methylethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Agria cactus	Week one Agria cactus rot	Week two Agria cactus rot	Week three Agria cactus rot	Week four Agria cactus rot
29	Isopentyl butyrate	0 ± 0	0.17 ± 0.14	0.11 ± 0.19	0.77 ± 0.37	0.62 ± 0.62
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
31	3-octanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
33	Acetoin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
34	n-propyl hexanoate	0 ± 0	0.43 ± 0.07	0.68 ± 0.77	2 ± 1.59	0.97 ± 1.14
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0 ± 0	0.04 ± 0.08	0 ± 0	0 ± 0
39	2-nonanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
42	Ethyl octanoate	0 ± 0	0.05 ± 0.09	0.31 ± 0.3	0.34 ± 0.09	0.26 ± 0.19
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
47	Propyl octanoate	0 ± 0	0.08 ± 0.15	0.15 ± 0.26	0.41 ± 0.37	0.51 ± 0.37
48	Linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
49	Benzaldehyde	0.03 ± 0.02	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	0 ± 0	2.77 ± 3.44	3.05 ± 3.1	3.22 ± 0.12	1.95 ± 1.18
51	Propionic acid	0 ± 0	0.82 ± 0.45	0.23 ± 0.39	0.57 ± 0.98	0 ± 0
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
54	Butyric acid	0 ± 0	43.38 ± 20.57	29.22 ± 20.1	37.66 ± 12.33	21.24 ± 16.08
55	Eugenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	0 ± 0	0 ± 0	7.54 ± 6.68	11.94 ± 0.71	7.54 ± 6.57
57	Acetophenone	0 ± 0	2.69 ± 4.66	0 ± 0	0 ± 0	0 ± 0
58	Ethyl benzoate	0 ± 0	6.45 ± 1.45	7.76 ± 2.54	7.48 ± 1.75	6.09 ± 2.31
59	Propyl benzoate	0 ± 0	8.99 ± 0.43	14.58 ± 4.09	13.24 ± 3.99	8.45 ± 5.98
60	Pentanoic acid	0 ± 0	1.17 ± 0.54	1.41 ± 1.32	0.89 ± 0.86	0 ± 0
61	Butyl benzoate	0 ± 0	0.47 ± 0.65	0.69 ± 0.19	1.04 ± 0.72	1.82 ± 1.81
62	Methyl salicylate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	2-phenethyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
65	2-methoxy phenol	0 ± 0	0.32 ± 0.28	0 ± 0	0 ± 0	0 ± 0
66	Hexanoic acid	0 ± 0	0.81 ± 0.24	1.54 ± 1.9	3.24 ± 2.83	1.78 ± 1.15

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Uninoculated Agria cactus	Week one Agria cactus rot	Week two Agria cactus rot	Week three Agria cactus rot	Week four Agria cactus rot
67	Phenethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
68	Isopentyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
69	Phenethyl alcohol	0 ± 0	0.43 ± 0.11	0.46 ± 0.05	0.35 ± 0.08	0.34 ± 0.23
70	Creosol	0 ± 0	0 ± 0	0.16 ± 0.27	0.11 ± 0.19	0.01 ± 0.02
71	Phenol	0 ± 0	1.39 ± 1.15	1.74 ± 0.76	1.71 ± 0.78	1.29 ± 1.15
72	4-ethylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
73	4-methyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
74	Octanoic acid	0 ± 0	0.32 ± 0.27	0.61 ± 0.26	0.48 ± 0.04	0.42 ± 0.38
75	4-ethyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Agria cactus rot	Week six Agria cactus rot	Week seven Agria cactus rot	Week eight Agria cactus rot	Week nine Agria cactus rot
1	Acetone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2	2-butanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	2-propanol	0.55 ± 0.96	0.48 ± 0.84	0.69 ± 0.99	1.55 ± 1.68	2.19 ± 0.28
4	Propyl acetate	1.22 ± 0.53	1.07 ± 0.78	0.5 ± 0.74	0.86 ± 0.75	2.16 ± 0.36
5	Isopropyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	Ethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	Isopropyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Propyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
9	6-methyl-2 Hepatanone	1.15 ± 0.61	0.81 ± 0.58	0.95 ± 1.09	1.04 ± 0.91	2.36 ± 0.67
10	Hexanal	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11	Ethyl butyrate	1.03 ± 0.44	0.78 ± 0.61	0.32 ± 0.33	0.55 ± 0.52	1.16 ± 0.36
12	Isopropyl isopentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	Isopropyl pentanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	1-propanol	2.29 ± 2.28	3.45 ± 2.47	1.86 ± 2.46	1.61 ± 2	5.47 ± 0.83
15	Isobutanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	Propyl butyrate	11.94 ± 2.54	6.1 ± 4.15	5.28 ± 5.54	7.75 ± 6.24	14.03 ± 2.98
17	Isopentyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	1-undecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	1-dodecene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	Butyl propionate	0 ± 0	0 ± 0	0 ± 0	0.3 ± 0.29	0 ± 0
21	Pentanoic acid 1-methylpropyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Isopentyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
23	2-heptanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	Butyl butyrate	0.9 ± 1.56	1.44 ± 1.92	2.04 ± 3.3	1.74 ± 2.82	3.55 ± 3.71
25	Isopentyl alcohol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
26	Hexanoic acid ethyl ester	0.43 ± 0.75	0.56 ± 0.96	0.24 ± 0.42	0.37 ± 0.6	0.52 ± 0.91
27	Hexanoic acid 1-methylethyl ester	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
28	Isopropyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Agria cactus rot	Week six Agria cactus rot	Week seven Agria cactus rot	Week eight Agria cactus rot	Week nine Agria cactus rot
29	Isopentyl butyrate	0 ± 0	0.32 ± 0.55	0.23 ± 0.26	0 ± 0	0 ± 0
30	2-heptanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
31	3-octanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	Hexyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
33	Acetoin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
34	n-propyl hexanoate	2.16 ± 3.21	0.79 ± 1.32	1.54 ± 1.97	1.64 ± 2.21	3.6 ± 2.93
35	Isobutyl tiglate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
36	Hexyl propionate	0 ± 0	0 ± 0	0 ± 0	0.66 ± 1.14	0 ± 0
37	2-methyl, 3-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
38	1-hexanol	0 ± 0	0 ± 0	0.04 ± 0.07	0 ± 0	0 ± 0
39	2-nonanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
40	Durenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
41	Butyl hexanoate	0 ± 0	0 ± 0	0.04 ± 0.07	0 ± 0	0 ± 0
42	Ethyl octanoate	0.29 ± 0.04	0.14 ± 0.13	0.24 ± 0.21	0.14 ± 0.12	0.24 ± 0.12
43	Isopentyl hexanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
44	2-octanol acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
45	N,N'-diethyl-1,3 benzenediamine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
46	2-nonanol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
47	Propyl octanoate	0.36 ± 0.32	0.02 ± 0.04	0.18 ± 0.25	0 ± 0	0.49 ± 0.42
48	Linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
49	Benzaldehyde	0 ± 0	0.01 ± 0.01	0 ± 0	0 ± 0	0 ± 0
50	Acetic acid	3.66 ± 2.84	3.46 ± 3.16	1.77 ± 1.56	2.05 ± 1.65	3.83 ± 2.56
51	Propionic acid	0 ± 0	0 ± 0	0.47 ± 0.81	0.47 ± 0.81	1.53 ± 0.65
52	n-propyl, 3-mercapto-propanoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
53	Methyl benzoate	0 ± 0	0 ± 0	0.1 ± 0.09	0 ± 0	0 ± 0
54	Butyric acid	25.06 ± 8.81	22.86 ± 16.34	17.74 ± 16.94	16.3 ± 13.41	28.05 ± 6.31
55	Eugenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
56	Isopropyl benzoate	8.16 ± 0.44	7.33 ± 3.4	4.73 ± 4.1	5.06 ± 3.06	10.09 ± 1.04
57	Acetophenone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
58	Ethyl benzoate	6.24 ± 1.08	4.39 ± 3.84	3.38 ± 3.01	3.35 ± 2.91	7.26 ± 1.3
59	Propyl benzoate	9.42 ± 3.16	5.57 ± 4.24	5.17 ± 3.78	4.56 ± 3.45	9.37 ± 2.85
60	Pentanoic acid	1.79 ± 1.59	1.01 ± 1.64	0.73 ± 0.7	1.1 ± 0.91	2.19 ± 0.84
61	Butyl benzoate	0.73 ± 0.79	0 ± 0	0.3 ± 0.53	0 ± 0	0 ± 0
62	Methyl salicylate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
63	2-tridecanone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	2-phenethyl acetate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
65	2-methoxy phenol	0 ± 0	0 ± 0	0.17 ± 0.15	0.36 ± 0.44	0.98 ± 0.86
66	Hexanoic acid	3.69 ± 3.67	1.06 ± 1.52	1.79 ± 2.31	1.71 ± 2.29	3.43 ± 3.56

Table S2. Relative amounts of volatile compounds in uninoculated and inoculated cacti. Volatile compounds (mean ± stdev) emitted from barrel, prickly pear, organ pipe and agria cacti, respectively.

	Compound	Week five Agria cactus rot	Week six Agria cactus rot	Week seven Agria cactus rot	Week eight Agria cactus rot	Week nine Agria cactus rot
67	Phenethyl propionate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
68	Isopentyl benzoate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
69	Phenethyl alcohol	0.36 ± 0.05	0.26 ± 0.23	0.19 ± 0.17	0.22 ± 0.14	0.56 ± 0.1
70	Creosol	0 ± 0	0.06 ± 0.1	0.11 ± 0.1	0.09 ± 0.07	0.26 ± 0.04
71	Phenol	1.22 ± 0.51	0.74 ± 0.45	0.78 ± 0.77	0.72 ± 0.64	1.29 ± 0.59
72	4-ethylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
73	4-methyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
74	Octanoic acid	0.49 ± 0.14	0.23 ± 0.2	0 ± 0	0 ± 0	0 ± 0
75	4-ethyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
76	2-methoxy-4-propyl phenol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
77	4-vinylguaiacol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table S3. Principal component values for volatile compounds in the four host cacti. Eigenvectors with highest scores are indicated in bold. The compounds which were present only once across all four cacti were excluded from the PCA.

		PC 1	PC 2	PC 3	PC 4
	Eigenvalue	11.17	6.14	5.64	4.35
	Percent variation	16.67	9.17	8.42	6.5
	Eigenvectors				
1	Acetone	**-0.15226**	-0.00047	-0.00702	-0.03604
2	2-butanone	-0.08536	-0.01274	-0.09704	**-0.13253**
3	2 propanol	0.18833	0.12607	0.07016	-0.03027
4	Propyl acetate	0.054	-0.11443	**0.14793**	0.16297
5	Isopropyl acetate	-0.06172	0.01305	0.0724	0.0549
7	Isopropyl propionate	-0.09111	0.04925	0.09293	0.06382
8	Propyl propionate	0.0741	**-0.30041**	0.10396	0.16899
9	2 Hepatanone-6-methyl	0.16395	0.19732	0.05887	-0.08688
10	Hexanal	0	0	0	0
11	Ethyl butyrate	0.11608	0.12472	-0.22076	0.17835
14	1 propanol	0.18336	**0.20222**	0.06682	-0.07062
15	Isobutanol	-0.03763	0.00385	-0.06987	0.01991
16	Propyl butyrate	**0.23966**	0.14197	0.10237	-0.03567
17	Isopentyl acetate	-0.11877	0.04524	0.09879	0.07218
18	1-undecene	-0.04885	0.0716	0.06029	0.12015
19	1-dodecene	0	0	0	0
20	Butyl propionate	0.04648	**-0.24057**	0.07452	0.07772
22	Isopentyl propionate	-0.10498	0.03614	0.07952	0.05025
23	2-heptanone	-0.03494	0.01715	0.02463	0.00376
24	Butyl butyrate	0.13302	0.18268	-0.17907	0.12966
25	Isopentyl alcohol	-0.11821	0.06262	-0.03083	0.04142
26	Hexanoic acid ethyl ester	0.13181	-0.01322	-0.16152	**0.22981**
27	Hexanoic acid 1-methylethyl ester	-0.00323	-0.06487	-0.16539	0.00763
28	Isopropyl tiglate	-0.04036	-0.02147	-0.06751	**-0.09703**
29	Isopentyl butyrate	0.06218	0.09121	**-0.28514**	**0.24756**
30	2-heptanol acetate	-0.06436	0.01829	0.06829	0.10127
31	3-octanone	-0.05919	0.02588	-0.01994	-0.01978
32	Hexyl acetate	-0.01417	-0.12534	0.07539	0.18671
33	Acetoin	-0.0938	0.07482	-0.0326	0.10478
34	n-propyl hexanoate	**0.19887**	-0.09242	0.09302	0.11495
36	Hexyl propionate	0.05114	-0.17666	0.06625	0.09265

38	1-hexanol	-0.02394	0.09305	0.00829	0.16031
39	2-nonanone	-0.13418	0.094	0.09093	0.12672
40	Durenol	-0.11164	0.10302	0.07656	0.14628
41	Butyl hexanoate	0.04138	0.04846	**-0.28867**	**0.26583**
42	Ethyl octanoate	**0.22212**	-0.04584	**0.12897**	0.06963
43	Isopentyl hexanoate	0.04143	0.04918	**-0.29398**	**0.27116**
45	N,N'-diethyl-1,3 benzenediamine	**-0.14839**	0.113	0.10076	0.13669
46	2-nonanol	-0.09605	0.10135	0.10516	0.17092
47	Propyl octanoate	0.1392	**-0.19144**	0.1113	0.11482
48	Linalool	-0.03716	-0.03815	**-0.26168**	**-0.09355**
49	Benzaldehyde	-0.06001	0.04485	0.02075	0.04721
50	Acetic acid	0.18757	**0.19907**	0.06044	-0.07669
51	Propionic acid	0.11916	**-0.23402**	0.10151	0.12677
53	Methyl benzoate	-0.13968	0.09016	0.05299	0.10007
54	Butyric acid	**0.24939**	0.06513	-0.03072	0.13701
55	Eugenol	-0.05883	0.01968	-0.04557	-0.01882
56	Isopropyl benzoate	0.19296	**0.2521**	0.01073	-0.02371
57	Acetophenone	0.00714	0.0121	-0.00345	-0.02824
58	Ethyl benzoate	0.15967	**0.27103**	0.07798	0.00393
59	Propyl benzoate	0.18452	**0.25143**	0.09324	-0.06709
60	Pentanoic acid	0.15397	0.02081	0.07624	0.00803
61	Butyl benzoate	0.09834	0.18151	0.05954	-0.05902
62	Methyl salicylate	-0.02469	0.03906	**-0.2849**	0.17138
63	2-tridecanone	**-0.15445**	0.10034	0.12574	0.14712
64	2-phenethyl acetate	-0.08434	0.01772	-0.04639	0.04839
65	2-methoxy Phenol	**-0.18379**	0.13963	**0.13795**	0.18359
66	Hexanoic acid	**0.19454**	-0.11227	-0.04193	**0.24369**
67	Phenethyl propionate	-0.11678	0.03555	0.06729	0.04815
68	Isopentyl benzoate	-0.08724	0.06157	-0.00938	0.03518
69	Phenethyl alcohol	-0.10033	0.04334	-0.13636	**-0.11149**
70	Creosol	-0.08706	0.03817	**0.13225**	0.10235
71	Phenol	-0.05028	0.17009	**0.14737**	0.07749
72	4-ethylguaiacol	-0.10716	0.08344	0.04778	0.106
73	4-methyl phenol	-0.04319	-0.04833	-0.14834	**-0.16518**
74	Octanoic acid	0.1627	**-0.18193**	0.11428	0.12793
75	4-ethyl phenol	-0.08213	-0.01259	-0.25463	-0.07991
76	2-methoxy-4-propyl phenol	-0.12883	0.09395	0.07663	0.12175
77	4-vinylguaiacol	**-0.15658**	0.10736	0.08025	0.15366

GENERAL DISCCUSION

Many insects are sensitive to volatile chemical signals that advertise relevant ecological information concerning nutrient resources, competitors, predators, potential mates, and host suitability (Price et al., 2011). The olfactory system of the vinegar fly, *Drosophila melanogaster*, serves as a genetically and anatomically simple model for studying how sensory input is translated into behavioral output, because only two synapses isolate the input to the peripheral detection system from the neurons governing the behavioral output. This genetic and anatomical simplicity of the *Drosophila* olfactory system have here allowed us to further our understanding of how olfactory systems operate.

Innate avoidance behavior

All animals exhibit innate behaviors in response to specific sensory stimuli, causing activation of developmentally preprogrammed neural circuits. This innate reaction to environmental stimuli enhances survival and reproduction (Price et al., 2011). Innate behaviors thus offer an excellent opportunity to explore how behaviors are organized in the nervous system and how they are programmed during development. An important first step toward this goal is to understand the neural pathways that mediate instinctive behaviors, from sensory input through to motor output.

Although the olfactory pathways that underlie hardwired attraction (Semmelhack and Wang, 2009; Min et al., 2013) and female courtship receptivity (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007; Grosjean et al., 2011) have been well characterized in *D. melanogaster*, the circuits that mediate odorant-induced innate avoidance are poorly understood. However, from an evolutionary perspective, being able to rapidly detect and respond to dangerous signals in the environment should be an essential task for the olfactory system. To date, there have been only three investigations on olfactory pathways that underlie innate avoidance behavior in *D. melanogaster*. The first study focused on the pathway that mediates the innate avoidance behavior towards carbon dioxide (CO_2). This atmospheric trace-gas is an important olfactory cue for many insects, including mosquitoes, flies, moths, and honeybees, in a variety of behavioral contexts (Guerenstein and Hildebrand, 2008; Hallem and Sternberg, 2008). In *Drosophila*, concentrations of CO_2 higher than 0.02% above atmospheric levels trigger avoidance behavior, which is mediated by a dedicated receptor channel (Suh et al., 2004; Faucher et al., 2006). CO_2 is exclusively detected by a unique

heteromeric receptor encoded by Gr21a and Gr63a (Clyne et al., 2000; Scott et al, 2001; Jones et al., 2007; Kwon et al., 2007) that is expressed in a single population of antennal olfactory sensory neurons (OSNs), called ab1C, short for antennal basiconic type 1 neuron C (Suh et al., 2004; Scott et al., 2007). These OSNs send axonal projections to the V glomerulus. Silencing this glomerulus blocks the avoidance response, and expressing channel rhodopsin in this OSN subpopulation causes flies to avoid blue light (Suh et al., 2004, 2007; Faucher et al., 2006). The ecological meaning of CO_2 for *Drosophila* is, however, unclear. CO_2 is present in significant quantities in essentially all important food sources that elicit behavioral attraction in flies. However, recent studies have showed that certain food odorants may directly inhibit the CO_2 response of ab1C neurons (Turner and Ray, 2009). In addition, strong activation of a neighboring OSN (ab1A) by food odorants may attenuate the response of the ab1C neuron through non-synaptic inhibition (Su et al., 2012).

The second investigation into the olfactory pathways that mediate avoidance behavior in the fly concerns DEET, or N,N-diethyl-meta-toluamide (Kain et al., 2013). This chemical is the primary insect repellent, and even though it has been use for more than 60 years, the exact mechanism by which this chemical causes repulsion has remained unclear. Using the nuclear factor of activated T cells (NFAT)-based system (Masuyama et al., 2012) to report DEET-evoked neural activity through expression of green fluorescent protein (GFP), the authors found an increase in GFP expression in a subset of OSNs that innervate sensilla within the sacculus, a pit like structure in the antennae. In the antennal lobe, the DEET-induced GFP signals were detected in the glomerulus innervated by axons of Ir40a-expressing OSNs. In addition, the Ir40a-expressing OSNs showed robust activation in response to DEET in *in vivo* calcium imaging experiments. Avoidance was also significantly decreased in flies that expressed the active form of the tetanus toxin (TNTG) from a Ir40a-Gal4 driver line as compared to an assortment of controls, suggesting that Ir40a-expressing OSNs are required for DEET repellency in flies. Using novel chemical informatics strategies, the authors moreover screened half a million compounds *in silico* against the DEET receptor and identified nearly 200 natural DEET substitutes. Of these, eight were strong repellents for flies. Finally, the authors found that Ir40a is conserved not only in Diptera, but also in many other insect lineages, including several human and plant pests. However, the ecological meaning of the DEET-detecting neural circuit for flies is still unclear; especially since DEET is not a naturally occurring compound. Future work will have to establish the identity of the natural ligand activating Ir40 and its role in insect ecology.

The third study describes a functionally segregated olfactory circuit in *D. melanogaster*, exclusively activated by geosmin (chapter I of this thesis (Stensmyr et al., 2012)). Geosmin represents an interesting candidate stimulus for a dedicated pathway for innate avoidance in flies, because previous work has shown that geosmin can suppress attraction of *Drosophila* to vinegar volatiles (Becher et al., 2010). Geosmin is produced by a number of fungi (Matteis and Roberts, 1992), bacteria (Gerber and Lechevalier, 1965), and cyanobacteria (Jüttner and Watson, 2007). Strikingly, our exhaustive SSR screen revealed that geosmin exclusively activated a single class of OSN - ab4B - which expresses the odorant receptor Or56a; a receptor for which no ligand had been identified in earlier screens (de Bruyne et al., 2001; Hallem et al., 2004). Axons from the OSNs of the ab4B OSNs target the DA2 glomerulus, which is situated in an area of the AL that has previously been shown to process negative hedonic valence (Knaden et al., 2012). Selective silencing of Or56a-expressing OSNs, via thermogenetics (Kitamoto, 2001) abolished the avoidance behavior to geosmin in attraction, feeding and oviposition experiments. Expressing the temperature sensitive dTRPA1 in Or56a neurons was also sufficient to cause avoidance. In addition, and importantly, thermogenetic silencing of Or56a neurons also caused flies to no longer avoid laying eggs in the presences of a harmful geosmin-producing microbe. Similarly, flies also laid eggs in a genetically modified strain of a geosmin producing microbe in which a key gene involved in the synthesis had been deleted. We furthermore found that the geosmin detection system was highly conserved across the genus *Drosophila*, suggesting that this circuit has a conserved function as a detector for the presence of toxic microbes. The evolutionary significance of this circuit is thus clear: it provides flies with a sensitive and specific mean to identify and avoid unsuitable hosts, or host resources that lower larval survival. The precision by which the fly's olfactory system detects geosmin only has parallels in the subset of the olfactory system that relays pheromone information. However, almost all pheromones characterized to date have been complex blends processed by multiple neuronal pathways. The discovery of an olfactory receptor that mediates innate avoidance to geosmin-producing pathogens engenders the question of how *Drosophila* detects other pathogens that do not produce geosmin. Future exploration of the chemical signals of non-geosmin producing pathogens may identify other hard-wired olfactory avoidance pathways, especially those related to the ecological function of several of the odorant receptors in *Drosophila* that are currently still elusive in revealing their best ligand.

Oviposition preference

Selecting a good oviposition site is a complex, important and challenging task. The oviposition strategy must take many factors into account, such as host range, clutch size, host quality, the difficulty of finding a host of sufficient quality, the chances of finding even better hosts, predation risks, larval mobility, host finding capability, microclimate, and so on (Janz 2002). In short, the process of choosing an optimal oviposition site is a daunting task for a small insect.

Drosophila melanogaster, which utilizes fermenting fruit as breeding substrate, chooses egg-laying sites after assessing a wide range of factors, including color (Volpe et al., 1967; del Solar and Ruiz 1979) surface texture of the substrate (David 1970; Takamura and Fuyama 1980), temperature (Fogelman 1979), light intensity (Wogaman and Seiger 1983), presence of eggs or other pre-adult stages (del Solar and Palomino 1966; del Solar 1968), ethanol and sugar content (McKenzie and Parsons 1972; Yang et al., 2008; Schwartz et al., 2012), fermentation volatiles (Reed 1938; Stökl et al., 2010), microbial composition (Stensmyr et al., 2012), as well as the substrate itself on which the microbes grow (i.e. the fruit) and the fruit produced volatiles (Dweck et al., 2013) as described in chapter II of this thesis.

Although the choice where to lay eggs is a complex behavior that relies upon input from multiple sensory modalities, the study in chapter II of this thesis has taken an important step in identifying the neurophysiological basis of this behavior. In a series of multiple-choice assay experiments, we allowed flies to oviposit on different types of fruit and found that flies displayed an overwhelming preference for non-acidic *Citrus* fruits. Flies clearly preferred intact oranges over peeled, implying that chemicals present in the flavedo were important. A key chemical in this context is limonene, and indeed, when confronted with transgenic oranges in which limonene production had been abolished, flies no longer displayed the oviposition preference. Presentation of limonene by itself triggered egg-laying, but no chemotaxis, indicating that this volatile indeed acts as a genuine oviposition stimulus. A system-wide single sensillum recording (SSR) screen from all OSN classes on the antennae and on the maxillary palps, stimulating with limonene revealed strong responses from only a single OSN type, the antennal intermediate sensillum type 2A (ai2A) neurons. These neurons express Or19a as revealed from the *in vivo* Ca imaging of the fly AL and the mis-expression of Or19a in Δab3A OSNs. Further testing of volatiles on the Or19a OSNs revealed that these neurons were specifically configured for the detection of terpenes. Thermogenetic experiment

also revealed that the Or19a OSNs were necessary and sufficient for the observed oviposition preference. The preference of *D. melanogaster*, an African species towards a fruit of Asiatic origin is somewhat enigmatic. Two possible scenarios might resolve it; *Citrus* may activate a preexisting olfactory bias, or the preference may be an ancestral trait from the Asian population of flies, which colonized Africa and later gave rise to the lineage leading to *D. melanogaster*.. Examining the responses of the ai2A neuron to *Citrus* volatiles in a variety of *Drosophila* species across the subgenus *Sophophora* showed that the Asian fly relatives exhibited responses similar to *D. melanogaster*. It is hence not inconceivable that the preference for *Citrus* and the tuning of the ai2A OSNs constitutes an ancestral trait that has remained conserved in the lineage leading to *D. melanogaster*. Another possibility is that the *Citrus* partiality reflects an ancestral preference towards specific fruit found within the native African habitat. Indeed, a search among native African fruits showed that fruits with chemistry and physical properties akin to that of citrus can be found in Africa. One fruit in particular, the African squirrel nutmeg, produced similar physiological and behavioral response as *Citrus*. Finally, we demonstrate that the preference for fruits with a thick rind, which characteristically causes terpene volatile emissions, might confer protection against endoparasitoid wasps, key enemies of flies. Our finding suggests that a limited number of olfactory pathways are involved in oviposition site selection.

Divergence in olfactory host preference

The olfactory system is an excellent system to study adaptive responses to altered environmental conditions and shifts in habitat preference because it is directly interfaced with the environment. Therefore, chapters III and IV of this thesis focused on the alterations in the olfactory machinery of *D. erecta* and *D. mojavensis*, relatives of *D. melanogaster*, that resulted from the association of *D. erecta* with *Pandanus* fruit and the divergence in host plant preference in response to cactus host utilization among the host races of *D. mojavensis*.

Drosophila erecta, the second specialized *Drosophila*-host system of the *melanogaster* species subgroup besides *D. sechellia* (that exclusively utilizes fruits of Noni *Morinda citrifolia*), is endemic to gallery forests of west-central Africa and specialized on the ripe fruits of *Pandanus* spp. Because the *Pandanus* trees fruit occurs only once a year over a period of about two months, *D. erecta* is considered to be a seasonal specialist (Rio et al., 1983). The aim of chapter III was to study changes in the olfactory system of *D. erecta* that resulted from the *D. erecta-Pandanus* association, which we studied by comparison with three

sympatric melanogaster sibling species (*D. melanogaster*, *D. yakuba*, and *D. orena*) each with different host specificity and ecology (Linz et al., 2013). Notably, few typical fruit-related compounds evoked very strong responses from the antennae of all four species. In a PCA analysis of the data, *D. erecta* measurements formed a discrete cluster, which did not overlap with its closest relative, *D. orena*. On the other hand, *D. erecta* grouped closer with *D. yakuba*, a species also attracted to *Pandanus* syncarps (Lachaise, 1974), which suggests that the recorded olfactory responses of the four species likely reflected lifestyle rather than phylogenetic relationship. The distinct clustering of the four species was mainly due to differential antennal responses to three main compounds, isoamyl acetate, 3-methyl-2-butenyl acetate and phenethyl alcohol. Isoamyl acetate and phenethyl alcohol are common natural fruit compounds (Stensmyr et al., 2003), while 3-methyl-2-butenyl acetate is rare in nature and has previously been reported as a diagnostic volatile of *Pandanus* syncarps (Vahirua-Lechat et al., 1996). This volatile specifically activates ab3A neurons and triggers oviposition in *D. erecta*, but not in *D. melanogaster*. Interestingly, this OSN subpopulation is also suspected to play a key role for *D. sechellia*'s specialization towards noni (Dekker et al 2005). Similar to *D. sechellia*, the percentage of the ab3 sensilla and the volume of their targeted glomerulus were also increased in *D. erecta* relative to *D. melanogaster*. Thus, these results not only support previous findings in the noni-fruit specialist *D. sechellia*, but also provide support for a general pattern of olfactory adaptations in insect-host associations.

Chapter IV focused on the divergence in the olfactory host plant preference among the four races of *D. mojavensis* in response to cactus host use. Understanding how reproductive isolation evolves requires a system in which there is phenotypic divergence among populations of the same species from contrasting environments and for which extensive ecological data have been collected (Via et al., 1999). *D. mojavensis* represents such a system, and is a model of incipient speciation. *D. mojavensis* originated in Baja California and invaded the deserts of mainland Mexico and southern California by a switch in host plant use (Heed. 1982; Heed and Mangan, 1986; Ruiz et al., 1990; Pfeiler et al., 2009). These geographically isolated populations show differing levels of premating isolation and no postmating isolation from one another (Heed, 1978; Zouros and d'Entremont, 1980 ; Ruiz et al., 1990; Knowles and Markow, 2001). Populations in Baja California [designated *D. m. baja*] use pitaya agria (*Stenocereus gummosus*), while mainland Mexico populations [*D. m. sonorensis*] use organ pipe cactus (*Stenocereus thurberi*), and populations in the Mojave desert [*D. m. mojavensis*] and on Santa Catalina Island [*D. m. wrigleyi*] use barrel cactus (*Ferocactus cylindraceous*) and prickly pear

cactus (*Opuntia spp.*), respectively (Heed and Mangan, 1986; Ruiz et al., 1990). The four populations of *D. mojavensis* feed and breed on four different species of fermenting cacti, so we began this study by measuring the attraction of each population to their respective host plants across a range of fermentation stages to determine the role of olfaction in host plant shifts. All four populations showed overall greater attraction to fermented (inoculated) rather than fresh (uninoculated) cactus tissue but the preferences for fermentation stage varied among the four populations. The Mojave Desert and S. Catalina populations showed clear preferences for earlier fermentation stages in accordance with previous studies (Downing, 1985), while the mainland Sonoran and Baja populations were equally attracted to all of the stages of fermentation. There is also an indication of sex specific responses and overall females tended to have stronger responses. These results are expected given previous findings in *Drosophila* that show differences in olfactory responses between the sexes and increased behavioral responses in *D. mojavensis* females relative to males (Reed, 1938; Fogleman, 1982; Newby and Etges, 1998). The fermentation-stage differences in preference arise because the fermentation-stages varied in the composition and abundance of volatiles produced. In general, the volatile blends of prickly pear and agria were primarily equal in number of esters and aromatics, but organ pipe and barrel cacti were enriched in esters and aromatics, respectively. With the notion that adaptation to different ecological environments can result in divergence of olfactory preference (Hansson and Stensmyr, 2011), we observed that the Mojave Desert population, specializing on barrel cactus, has diverged in its peripheral odor detection machinery with an overall decreased response to esters and increased response to aromatics, similar to intraspecific variation in the odor-guided behavior that has been observed previously in the tephritid fly *Rhagoletis pomonella*, where differences were also due to divergence in the peripheral sensitivity among populations (Olsson et al., 2006a and 2006b). These electrophysiological differences most likely reflect alterations in ligand binding or receptive range of the OSNs, either through amino acid substitutions in chemosensory receptors as these have been shown to confer differences in odorant sensitivity (Krautwurst et al., 1998; Abaffy et al., 2007; Keller et al., 2007), or changes in the number of OSNs as in the case of *D. erecta* (Linz et al., 2013) and *D. sechellia* (Dekker et al., 2006). The estimates of divergence between *D. mojavensis* and *D. arizonae* range between 1.91 and 2.97 million years ago (Matzkin and Eanes, 2003; Reed et al., 2007), and this short time span suggests a rapid adaptation to the changes in host plant utilization of this plant-insect system. Our findings will help unravel the mechanisms underlying the process of species formation and the evolution of host-plant specialization.

Concluding remarks

In summary, we identified two olfactory pathways in the vinegar fly; one that underlies hardwired innate avoidance of harmful microbes and a second pathway that mediates oviposition site preference. We also showed that the peripheral odor detection machinery of *D. erecta* and *D. mojavensis* have been modified to match the olfactory needs of their respective ecological niches and associated host plants. The findings of this thesis, while most directly pertinent to the advancement of our understanding of the basic science behind insect olfactory mechanisms, may also form a base for future developments of integrated pest management strategies for troublesome insects.

GENERAL SUMMARY

The genetic and anatomical simplicity of the *Drosophila melanogaster* olfactory system allows us to identify two distinct olfactory pathways, one that underlies a hardwired innate avoidance behavior and a second pathway that governs oviposition preference. The first pathway is a functionally segregated olfactory circuit, transferring the message arising from the periphery unaltered to the central processing centers. This olfactory circuit is activated exclusively by the microbial odor, geosmin, a cue which serves to alert flies to the presence of harmful microbes. Geosmin activates only one class of olfactory sensory neuron, which expresses the odorant receptor, Or56a, for which no ligand had previously been identified. These neurons in turn target the DA2 glomerulus and connect to projection neurons that respond exclusively to geosmin. Selectively silencing Or56a-neurons abolishes the observed avoidance behavior to geosmin, and suppresses the aversive influence of this compound on feeding. Furthermore, these Or56a silenced flies lay eggs upon medium containing the geosmin producing *S. coelicolor* as well as uncontaminated medium. Expressing the temperature sensitive dTRPA1 in Or56a neurons is also sufficient to make flies to avoid high temperature (T >26°C). Geosmin also overrides and modulates innate attraction to vinegar, a substance which confers obligate attraction in normal, wild type flies. Moreover, the geosmin detection system was highly conserved across virtually all species in the genus *Drosophila*, suggesting that the circuit evolved to successfully enable general avoidance of toxic feeding and breeding sites in the environment. On the other hand, the second pathway studied in this thesis deals with the olfactory preference for oviposition on *Citrus* substrates. Flies detect the terpenes that are characteristic of the *Citrus* fruits again via only a single class of olfactory sensory neurons, which in this case express the odorant receptor Or19a. These OSNs are both necessary and sufficient for this behavioral preference to occur, as revealed by silencing and artificial activation of these OSNs-Or19a. This preference towards citrus likely reflects an ancestral preference towards specific fruit found in their native African habitat. It has, moreover, likely been driven by the need to avoid parasitism from endoparasitoid wasps, since the same terpene ligands that mediate fly oviposition are also potent repellents for parasitic wasps that prey on fly larvae.

We also conducted extensive studies of olfactory adaptation resulting from the *D.erecta-Pandanus* association. In this case, we found that the adaptation occurs at two levels. First, at the periphery, with increased number of a specific input channel, the ab3 sensillum, and this

detects the diagnostic *Pandanus* fruit volatile, 3-methyl-2-butenyl acetate. At the second level, the adaptations from the periphery are also reflected in the changes to the AL morphology, specifically the enlargement of the DM2 glomerulus.

In addition, we have begun to understand the evolution of the olfactory changes in response to host plant shifts in the desert species, *D. mojavensis*. In this case, we observed that the Mojave Desert population, specializing on barrel cactus, has diverged in its peripheral odor detection machinery with an overall decreased response to esters and an increased response to aromatics. This divergence from the other three populations coincides with the fact that the volatiles released by fermenting barrel cactus contain a high abundance of aromatics when compared to the other three host cacti. The estimates of divergence between *D. mojavensis* and *D. arizonae* range between 1.91 and 2.97 million years ago, and this short time span suggests a rapid adaptation to the changes in host plant utilization of this plant-insect system. Our findings in this *D. mojavensis* ecological system will help unravel the mechanisms underlying the process of species formation and the evolution of host-plant specialization.

In summary, we have shown that the vinegar fly, *Drosophila melanogaster*, has two olfactory pathways, one that underlies the hardwired innate avoidance of harmful microbes and a second pathway for oviposition in *Citrus* substrates. We also showed that the peripheral odor detection machinery of *D. erecta* and *D. mojavensis* have been modified to match the olfactory needs of their respective ecological systems and associated host plants. Thus the findings of this thesis, while most directly pertinent to the advancement of our understanding of the basic science behind olfactory mechanisms, may also be useful in integrated pest management strategies for these and other potential pest insects.

ZUSAMMENFASSUNG

Das olfaktorische System der Essigfliege *Drosophila melanogaster* ist im Vergleich zu den Riechsystemen anderer Tiermodelle sehr einfach aufgebaut. Daher und aufgrund der zahlreichen etablierten genetischen Werkzeuge (die z.B. das An- bzw. Abschalten spezifischer Neuronenpopulationen ermöglichen) war es uns möglich, die Detektion und zentralnervöse Verschaltung von zwei ökologisch relevanten Düften im Detail zu untersuchen. Der erste Duft, Geosmin, ist ein Signalduft, der Fliegen auf die Anwesenheit gefährlicher Mikroben hinweist. Fliegen steuern attraktive Futterdüfte nicht mehr an, sobald ihnen Geosmin beigemischt wird. Ausserdem legen sie keine Eier an Substrat ab, das mit Geosmin versetzt wurde. Ich konnte zeigen, dass Geosmin von den Fliegen nur mit den olfaktorischen sensorischen Neuronen (OSNs) detektiert wird, die den Duftrezeptor Or56a exprimieren. Gleichzeitig reagieren diese OSNs ausschliesslich auf diesen Duft. Geosmin-sensitive Neuronen projizieren im ersten Duftneuropil, dem Antennallobus, in den sogenannten Glomerulus DA2. Dort sind sie mit Projektionsneuronen verknüpft, die widerum ausschliesslich von diesen OSNs Informationen erhalten. Selektives Ausschalten der Or56a-exprimierenden OSNs bewirkte, dass die Fliegen Geosmin nicht mehr vermieden. Weiterhin war ich in der Lage durch den genetischen Einbau eines temperatursensitiven Proteins (dTRPA1) in die Or56a-exprimierenden OSNs, diese künstlich durch Temperatur zu erregen. Fliegen mit diesem genetischen Konstrukt vermieden erhöhte Temperatur (d.h. die Temperatur, bei der ihnen aufgrund der künstlichen Aktivierung des OSNs die Anwesenheit von Geosmin vorgetäuscht wurde). Somit ist die Aktivierung von Or56a exprimierenden OSNs notwendig und ausreichend, um das Geosmin-spezifische Vermeidungsverhalten auszulösen. Interessanterweise verfügen fast alle Arten der Gattung *Drosophila* über diesen Rezeptor, was auf die ökologische Relevanz der Vermeidung von durch Mikroben verunreinigter Nahrung und Eiablageplätze hinweist.

Im Gegensatz zu Geosmin sorgen Terpene, welche vermehrt von Zitrusfrüchten gebildet werden, dafür dass *Drosophila* Zitrusfrüchte gegenüber anderen Früchten bevorzugt. Erneut detektieren die Fliegen diese Stoffgruppe mit einem spezielen Neuronentyp, den Or19a-exprimierenden OSNs. Durch gezieltes An- und Abschalten, konnte ich widerum zeigen, daß die Aktivierung dieser OSN-Population notwendig und ausreichend ist, um die gezeigte Präferenz für Zitrusfrüchte zu erklären. Da Zitrusfrüchte nicht in dem ursprünglichen Habitat von *Drosophila melanogaster* vorkommen, resultiert die Präferenz wahrscheinlich aus einer Anpassung an eine lokale Frucht, die obwohl nicht näher mit Zitrusfrüchten verwandt, über

ein ähnliches Duftspektrum und eine ähnliche Fruchtschale verfügt. Interessanterweise sind die von den Früchten abgegebenen Terpene zwar für die Fliegen attraktiv, schrecken jedoch parasitierende Wespen ab, die für die Fliegenlarven normalerweise eine große Gefahr darstellen.

Weiterhin konnte ich bei einer anderen *Drosophila* Art (*D. erecta*) zeigen, dass diese besonders sensitiv auf einen von ihrer Wirtspflanze abgegebenen Duft (3-methyl-2-butenyl acetate) reagiert. Diese morphologische Spezialisierung für diesen Duft hat auf zwei Ebenen stattgefunden: zum einen haben *D. erecta* Fliegen mehr ab3-Sensillen (welche für die Detektion des Duftes zuständig sind). Zum anderen ist im Antennallobus der Glomerulus (DM2), der von den involvierten OSNs innerviert wird, stark vergrößert.

In einer weiteren Studie habe ich die Artbildung von *D. mojavensis* und *D. arizonae* untersucht. Erstere besiedelt eine bestimmte Kaktusart, die sich durch die Produktion vieler aromatischer Komponenten in ihrem Duftspektrum auszeichnet. Erneut zeigte sich, daß schon auf der Peripherie des Duftsystems der Fliege Veränderungen stattgefunden haben, die eine erhöhte Sensitivität für die Wirts-spezischen Düfte ermöglicht. Die von uns gefundenen sensorischen Unterschiede der beiden Fliegenarten,und die Tatsache, daß die phylogenetische Trennung von *D. mojavensis* und *D. arizonae* vor nur etwa 1,91 bis 2,97 Millionen Jahren stattgefunden hat, zeigt, wie schnell sich das olfaktorische System an durch Wirtswechsel bedingte geänderte Anforderungen anpassen kann.

Zusammenfassend konnte ich zeigen, daß die Essigfliege *Drosophila melanogaster* über spezialisierte neuronale Versschaltungen für diverse ökologisch relevante Düfte verfügt, die für angeborene Verhaltensweisen verantwortlich sind. Weiterhin konnte ich zeigen, daß sich die sensorische Peripherie von *D. erecta* und *D. mojavensis* an die ökologischen Anforderungen angepasst hat. Obwohl die generellen Fragestellungen meiner Dissertation gundlegender Natur waren, könnten sie letztendlich für die Bekämpfung schädlicher oder störender Insektenarten hilfreich sein.

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DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscript. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller University Jena or to any other university.

Jena, January 17, 2014

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